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(54) Alcohol acetyltransferase genes and use thereof.

(57) This invention disclosed herein provides an alcohol acetyl transferase ("AATase"), an AATase encoding gene and a yeast having an improved ester producing ability due to transformation with the AATase encoding gene. This invention also provides a process for producing an alcoholic beverage having an enriched ester flavor using the transformed yeast.

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Description

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to an alcohol acetyltransferase ("AATase") produced by, for example, Saccharomyces cerevisiae, a DNA sequence encoding, i.e., having an ability for biotechnologically producing, AATase, and a yeast having an improved ester producing ability due to the transformation with the DNA sequence. The present invention also relates to a process for producing an alcoholic beverage having an enhanced ester flavor.

Related Art

It is well known that acetate esters affect the flavor quality of alcoholic beverages such as sake, beer, wine and whisky. These esters are in general present in the fermented supernatant, because yeast produces a various kinds of alcohols which are further converted into esters during a fermentation procedure.

In particular, isoamyl acetate is an ester which provides a good fruity flavor for alcoholic beverages. It has been suggested that the ratio of isomyl acetate to isoamyl alcohol, which is a precursor of isomyl acetate, is closely related to the evaluation value of the sensary test. For example, sake having a great ratio of isomyl acetate to isoamyl alcohol valued as "Ginjo-shu" in the sensary test (JOHSHI HOKOKU, No. 145, P. 26 (1973)).

AS previously reported by Yoshioka et al., Agric. Biol. Chem., 45, 2188 (1981), AATase is an enzyme which plays primary role in the production of isoamyl acetate. The AATase synthesizes isoamyl acetate by the condensation of isoamyl alcohol and acetyl-CoA. Furthermore, AATase has been known to have a wide substrate specificity and to produce many acetate esters such as ethyl acetate in the same mechanism as described above.

Therefore, in order to increase the esters, such as isoamyl acetate in the alcoholic beverages, it is effective to enhance the AATase activity of a yeast. Some of the conventional consideration in the production of the alcoholic beverages, for example, selecting raw materials or controlling fermentation conditions, as a result, have enhanced the activity of the AATase.

However, though it has been well known that AATase is important enzyme for the production of esters, there are few reports referring to the AATase. Partial purifications of the enzyme have been described in some reports (for example, NIPPON NOGEI KAGAKUKAISHI, 63, 435 (1989); Agric. Biol. Chem., 54, 1485 (1990); NIPPON JOZO KYOKAISHI, 87, 334 (1992)), but, because AATase has very labile activity, complete purification of AATase, and the cloning of the gene encoding AATase has not been reported, so far.

SUMMARY OF THE INVENTION

An object of the present invention is to reveal the structure of AATase and isolate the AATase gene, thereby to obtain a transformed yeast having an enhanced AATase producing ability and to produce an alcoholic beverage having an enhanced ester flavor.

According to the first embodiment of the present invention, the present invention provides an AATase originated from yeast having an ability for transferring the acetyl group from acetyl-CoA to an alcohol to produce an acetate ester and having a molecular weight of approximately 60,000 by SDS-PAGE.

According to the second embodiment of the present invention, the present invention provides an AATase comprising a polypeptide selected from a group consisting of:
(1a) a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;
(1b) a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 2; and
(1c) a polypeptide having an amino acid sequence from A to C or B to C of the amino acid sequence shown in Fig. 17.

According to the third embodiment of the present invention, the present invention provides the AATase encoding gene having a DNA sequence selected from a group the consisting of:
(2a) a DNA sequence encoding a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;
(2b) a DNA sequence encoding a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 2; and
(2c) a DNA sequence encoding a polypeptide having an amino acid sequence from A to C or B to C of the amino acid sequence shown in Fig. 17.

According to the fourth embodiment of the present invention, the present invention provides a DNA sequence comprising an AATase gene selected from a group consisting of:
(3a) an AATase gene having a DNA sequence from A to B of the DNA sequence shown in Fig. 1;
(3b) an AATase gene having a DNA sequence from A to B of the DNA sequence shown in Fig. 2;
(3c) an AATase gene having a DNA sequence from A to C or B to C of the DNA sequence shown in Fig. 17; and
(3d) a DNA sequence which hybridizes with any one of genes (3a) to (3c).

According to the fifth embodiment of the present invention, the present invention provides a transformed yeast having an enhanced AATase producing ability due to the transformation using the AATase gene selected from (2a) to (2c) or a DNA sequence selected from (3a) to (3d).

According to the sixth embodiment of the present invention, the present invention provides a process for producing a alcoholic beverage having an enriched ester flavor using a transformed yeast as described above.

According to the seventh embodiment of the present invention, the present invention provides a method for isolating a DNA sequence encoding AATase, comprising the steps of:
(a) preparing a DNA fragment having a length of at least 20 bases of a DNA sequence which encodes a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;
(b) preparing a gene library which has been made from DNA strands having a substantially same length in the range from 5 x 10 to 30 x 10 bases obtained by cutting the chromosome of a yeast;
(c) cloning a DNA fragment by hybridization from gene library of (b), using the DNA fragment of (a) as a probe.

The terms "DNA fragment", "DNA sequence" and "gene" are herein intended to be substantially synonymously.

Since the AATase gene have been obtained, a yeast can be transformed using this gene as a foreign gene by a genetic engineering method. That is, the gene can be transfected into a yeast cell as a extranuclear and/or intranuclear gene to afford the yeast an AATase producing ability greater than that of the host cell, and using these transformants an alcoholic beverage having the enriched ester flavor can be made.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 (a) and (b) show an amino acid sequence of AATase and DNA sequence of the AATase encoding gene according to the present invention;
Figs. 2 (a) and (b) show a amino acid sequence of AATase and DNA sequence of another AATase encoding gene according to the present invention;
Fig. 3 shows a restriction map of the AATase encoding gene originated from a sake yeast according to the present invention;
Fig. 4 shows two restriction maps of the AATase originated from a brewery lager yeast according to the present invention;
Fig. 5 shows a restriction map of the AATase originated from a wine yeast according to the present invention;
Fig. 6 shows the process for preparing the probe used for obtaining the AATase gene from the wine yeast;
Fig. 7 shows the elution profile of an AATase active fraction by the affinity chromatography method among the purification processes according to the present invention;
Fig. 8 shows an SDS-polyacrylamide electrophoresis of the AATase active fraction eluted by the affinity chromatography according to the present invention;
Fig. 9 shows the substrate specificity of the AATase according to the present invention to a variety of alcohols;
Fig. 10 shows a restriction map of the expression vector YEp13K for yeast;
Fig. 11 shows a restriction map of the expression vector YATK11 having the AATase gene originated from a sake yeast according to the present invention;
Fig. 12 shows a restriction map of the expression vector YATL1 having the AATase 1 gene originated from a brewery lager yeast according to the present invention;
Fig. 13 shows a restriction map of the expression vector YATL2 having the AATase 2 gene originated from a brewery lager yeast according to the present invention;
Fig. 14 shows a restriction map of the sake-yeast expression vector YATK11G having the AATase gene originated from a sake yeast according to the present invention;
Fig. 15 shows a restriction map of the brewery lager yeast vector YATL1G having the AATase 1 gene originated from a brewery lager yeast according to the present invention;
Fig. 16 shows a part of the brewery lager yeast expression vector construction; and
Figs. 17 (a) and (b) shows the amino acids and DNA sequence of the brewery lager yeast AATase 2 gene according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

AATase

AATase, alcohol acetyltransferase, is an enzyme having an ability for producing an acetate ester by transferring the acetyl group from acetyl-CoA to alcohols.

The alcohols herein primarily mean alcohols having straight or branched chains having 1 to 6 carbon atoms. According to our studies, however, it has been found that the AATase may employ as substrates alcohols having a higher number of carbon atoms such as 2-phenyl ethylalcohol.

Thus, "the alcohols" should be construed to include a wide range of alcohols, if it is necessary to discuss the substrate alcohol of the AATase in the present invention.

The AATase according to the present invention is originated from yeast. The AATase is specifically obtained from Saccharomyces cerevisiae and is a polypeptide having any one of the polypeptides (1a) - (1c) defined above. Specifically, the polypeptide includes a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1; a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 2; a polypeptide having an amino acid sequence from A to C of the amino acid sequence shown in Fig. 17; and a polypeptide having an amino acid sequence from B to C of the amino acid sequence shown in Fig. 17.Furthermore, it has been clarified by genetic engineering or protein engineering that the physiological activity of a polypeptide may be maintained with the addition, insertion, elimination, deletion or substitution of one or more of the amino acids of the polypeptide. The polypeptide therefore include a modified polypeptide of any one of the above polypeptides due to the addition, insertion, elimination, deletion or substitution of one or more of amino acid of the polypeptide so long as the modified polypeptide has an AATase activity.

Saccharomyces cerevisiae used herein is a microorganism described in "The yeast, a taxonomic study", the 3rd Edition, (ed. by N.J.W. Kreger-van Rij, Elsevier Publishers B.V., Amsterdam (1984), page 379), or a synonym or mutant thereof.

AATase and its purification method have been reported in some papers, for instance, NIPPON NOGEIKAGAKU KAISHI, 63, 435 (1989); Agric. Biol. Chem., 54, 1485 (1990); NIPPON JOSO KYOKAISHI, 87, 334 (1992). However, so far as the present inventors know, the AATase has not been purified to homogeneity, so its amino acid sequence has not been determined.

The present inventors have now found that an affinity column with 1-hexanol as a ligand can be used successfully for purifying the AATase. We have thus completely purified the AATase from Saccharomyces cerevisiae by use of this affinity column and defined some properties of the enzyme. The amino acid sequence shown in Fig. 1 is obtained by analysis of the AATase originated from Saccharomyces cerevisiae which has thus purified to homogenity.

The typical property of the AATase which have been defined according to the present invention includes the molecular weight of the AATase. Although the molecular weight of the AATase previously reported is in the range from 45,000 to 56,000, the molecular weight of the AATase purified according to the present invention is approximately 60,000 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), suggesting that it is different from the protein reported previously. The molecular weight of the AATase deduced from the DNA sequence was ca. 61,000.

The AATase of the present invention has enzymological and physicochemical properties as set forth below.
(a) Action:
This enzyme acts on a variety of alcohol such as ethyl alcohol and acetyl-CoA to produce an acetate ester.
(b) Substrate specificity:
This ensyme acts on various kinds of alcohol having 2 to 5 carbon atoms, more efficiently on alcohols having 2 to 5 carbon atoms.In addition, the enzyme acts more efficiently on straight chain alcohols rather branched chain alcohols.
(c) Molecular weight: ca. 60,000
(d) Optimum and stable pH:
optimum pH: 8.0,
stable pH: 7.5 - 8.5
(e) Optimum and stable temperature:
optimum temperature: 25 DEG C,
stable temperature: 4 DEG C;
(f) Inhibitors:
This enzyme is intensively inhibited by parachloromercury benzoate (PCMB) and dithiobisbenzoic acid (DTNB);
(g) Effects of various fatty acids on the activity:
This enzyme is not noticeably inhibited by a saturated fatty acid but intensively inhibited by an unsaturated fatty acid;
(h) Km value to isoamyl alcohol and acetyl-CoA:
isoamyl alcohol: 29.8 mM,
acetyl CoA: 190 mu M.

The AATase can be obtained by a procedure comprising culturing yeast cells of Saccharomyces cerevisiae KYOKAI No. 7 and recovering and purifying the crude enzyme from the content of the organism as described in Examples below.

DNA sequence or DNA fragment/gene which produces AATase

In the present invention, the DNA sequence or DNA fragment having an ability of producing AATase means the DNA sequence or DNA fragment which codes for a polypeptide having AATase activities. The amino acid sequence of a polypeptide encoded by the sequence or fragment, i.e., the AATase, is selected from the group consisting of the following (2a) - (2c), and is specifically selected from the group consisting of the following (3a) - (3d):
(2a) a DNA sequence encoding a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;

(2b) a DNA sequence encoding a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 2;
(2c) a DNA sequence encoding a polypeptide having an amino acid sequence from A to C or B to C of the amino acid sequence shown in Fig. 17.
(3a) an AATase gene having a DNA sequence from A to B of the DNA sequence shown in Fig. 1;
(3b) an AATase gene having a DNA sequence from A to B of the DNA sequence shown in Fig. 2;
(3c) an AATase gene having a DNA sequence from A to C or B to C of the DNA sequence shown in Fig. 17; and
(3d) a DNA sequence capable of hybridizing with any one of genes (3a) to (3c).

The DNA sequence varies depending upon the variation of the polypeptide. In addition, it is well known by one skilled in the art that a DNA sequence is easily defined according to the knowledge referring to the so called "degeneracy", once an amino acid sequence is given. Thus, one skilled in the art can understand that certain codons present in the sequence shown in Figs. 1, 2 and 17 can be substituted by other codons and produce a same polypeptide. This means that the DNA sequence (or DNA fragment) of the present invention includes DNA sequences which encode the same peptide but are different DNA sequences in which codons in the degeneracy relation are used.Furthermore, one skilled in the art can understand that the DNA sequence of the present invention include the DNA sequence which encodes a modified polypeptide of any of one of the polypeptides (1a) to (1c) due to the addition, insertion, elimination, deletion or substitution of one or more amino acid of these polypeptides. In this connection, the term "encoding" is synonymous with the term "capable of encoding".

The DNA sequence of the present invention may be obtained from a natural gene source or obtained by total synthesis or semi-synthesis (i.e., synthesized with use of a part of a DNA sequence originated from a natural gene source).

Form the natural gene source, the DNA sequence of the present invention can be obtained by conducting DNA manipulations such as plaque hybridization, colony hybridization and PCR process using a probe which is a part of a DNA sequence producing the AATase of the present invention. These methods are well-known to one skilled in the art and can be easily performed.

Suitable gene sources for obtaining a DNA sequence having an AATase producing ability by these methods include for example bacteria, yeast and plants. Among these gene sources, yeast which is currently used for the production of fermentation foods such as sake and soy sauce is one of the best candidate having a DNA sequence of the present invention.

The typical form of the DNA sequence of the present invention is a polypeptide which has a length just corresponding to the length of AATase. In addition, the DNA sequence of the present invention may have an additional DNA sequences which are bonded upstream and/or downstream the sequence. A specific example of the latter is a vector such as plasmid carrying the DNA sequence of the present invention.

Suitable example of the DNA sequence of the present invention is from A to B of the amino acid sequence shown in Fig. 1. This sequence is obtained by analyzing an AATase encoding gene obtained from a yeast strain, SAKE YEAST KYOKAI No. 7.

Transformation

The procedure or method for obtaining a transformant is commonly used in the field of genetic engineering. In addition to the method described below, any conventional transformation method (for example, Analytical Biochemistry, 163, 391 (1987)), is useful to obtain the transformant.

Vectors which can be used include all of the known vectors for yeast such as YRp vectors (multicopy vectors for yeast containing the ARS sequence of the yeast chromosome as a replication origin), YEp vectors (multicopy vectors for yeast containing the replication origin of the 2 mu m DNA of yeast), YCp vectors (single copy vectors for yeast containing the DNA sequence of the ARS sequence of the gene chromosome and the DNA sequence of the centromere of the yeast chromosome), YIp vectors (integrating vectors for yeast having no replication origin of the yeast). These vectors is well-known and described in "Genetic Engineering for the Production of Materials", NIPPON NOGEI KAGAKUKAI ABC Series, ASAKURA SHOTEN, p.68, but also can be easily prepared.

In addition, in order to express the gene of the DNA sequence according to the present invention or to increase or decrease the expression, it is preferable that the expression vector contains a promoter which is a unit for controlling transcription and translation in the 5'-upstream region and a terminator in the 3'-downstream region of the DNA sequence. Suitable promoters and terminators are for example those originated from the AATase gene itself, those originated from any known genes such as alcohol dehydrogenase gene (J. Biol. Chem., 257, 3018 (1982)), phosphoglycerate kinase gene (Nucleic Acids Res., 10, 7791 (1982)) or glycerolaldehyde-3-phosphate dehydrogenase gene [J. Biol. Chem., 254, 9839 (1979)) or those which are the artificial modifications of the former.

The yeast to be transformed in the present invention, i.e. the host yeast, may be any yeast strain which belongs taxonomically to the category of yeast, but for the purpose of the present invention, a yeast strain for producing alcoholic beverages which belongs to Saccharomyces cerevisiae such as brewery yeast, sake yeast and wine yeast are preferred. Suitable examples of yeast include brewery yeast such as ATCC 26292, ATCC 2704, ATCC 32634 and AJL 2155; sake yeast such as ATCC 4134, ATCC 26421 and IFO 2347; and wine yeast such as ATCC 38637, ATCC 38638 and IFO 2260.

Another group preferred as the host yeast is baker's yeast such as ATCC 32120.

Preparation of Alcoholic Beverages

The transformed yeast having an enhanced AATase producing ability is provided with a character intrinsic to the host yeast as well as the introduced character. The transformant thus can be used for various applications focussed to the intrinsic character.

If the host yeast is a yeast for preparing alcoholic beverages, the transformed yeast also has an ability for fermenting saccharides to alcohols. Therefore, the transformed yeast according to the present invention provides an alcoholic beverages having an enhanced or enriched ester flavor.

Typical alcoholic beverages include sake, wine, whiskey and beer. In addition, the process for preparing these alcoholic beverages are well-known.

Production of Other AATases

As described above, the present invention provides the AATase gene encoding amino acid sequence from A to B of the amino acid sequence shown in Fig. 1. According to another aspect of the present invention, the present invention provides other AATase genes. It has now been found that a different kind of AATase producing gene is obtained from a yeast gene library by use of a probe which is a relatively short DNA fragment of a DNA sequence encoding the amino acid sequence from A to B of the amino acid sequence shown in Fig. 1. It is interesting in this case that the probe originated from the DNA sequence obtained from a "sake" yeast provided two different DNA sequences having an AATase producing ability from the gene library of a brewery lager yeast.In addition, while both of these DNA sequences are capable of producing AATase, the restriction maps, DNA sequences and the amino acid sequences of the DNA sequences are different from those of the amino acid sequence shown in Fig. 1 originated from a sake yeast.

In the process of isolating these DNA sequences, a DNA fragment as a probe is first provided. The probe has preferably a length of at least 20 bases of the DNA sequence encoding a polypeptide having an amino acid from A to B of the amino acid sequence substantially shown in Fig. 1.

The length of the DNA strand as the probe is preferably at least 20 bases, since sufficient hybridization will not occur with an excessively short probe. The DNA strand has more preferably a length of 100 bases or more.

The gene library to which the probe is applied preferably comprises vectors containing DNA fragments having a substantially same length in the range from 5 x 10 bases to 30 x 10 bases obtained by cutting a chromosome of yeast by chemical or physical means such as restriction enzyme or supersonic.

The restriction enzyme to be used in this procedure, of which the kinds and/or the reaction conditions should be set up so that for a certain yeast chromosome the DNA strands having a length within the above range are obtained. In case of making gene library from brewery yeast chromosommal DNA, suitable restriction enzymes include for example Sau3AI or MboI.

It is desirable that the DNA fragment obtained by cutting have substantially the same length in the range from 5 x 10 bases to 30 x 10 bases, in other words, the DNA fragment in the digested product with restriction enzyme has uniform length within the range from 5 x 10 bases to 30 x 10 bases.

Cloning of the complementary DNA strands from the gene library using probes, and the subcloning of this cloned DNA fragments, for example, into the yeast is easily performed according to the well-known genetic engineering method (for example, Molecular Cloning, Cold Spring Harbor Laboratory (1988)).

The amino acid sequence shown in Fig. 2 is a polypeptide encoded by one of the two DNA sequences obtained from the brewery lager yeast gene library by using a probe which has a sequence corresponding to the DNA sequence from 234 to 1451 shown in Fig. 1. It is apparent from comparing the figures, the AATases originated from brewery lager yeast and sake yeast, are different from each other only in 12 base pairs and 3 amino acids. The polypeptide having an amino acid sequence from A to B shown in Flg. 2 which was obtained with the hybridization/cloning method described above, can also be regarded as an equivalent polypeptide of the amino acid sequence from A to B shown in Fig. 1, i.e., as a modified polypeptide in which some of amino acids have been deleted, substituted or added.

Similarly, the amino acid sequences (from A to C or from B to C) shown in Fig. 17 is polypeptides encoded by the other DNA sequence obtained from the gene library of brewery larger yeast by using the some probe. It is apparent from comparing the figures, this AATase originated from brewery lager yeast is different from the AATase originated from sake yeast in 332 base pairs and 102 amino acids.

Examples

The following examples are offered by way of illustration and are not intended to limit the invention any way. In the Examples, all percentages are by weight unless otherwise mentioned.

(1) Preparation of AATase

The enzyme of the present invention can be obtained from the culture of an microorganism which is a member of Saccharomyces and produces an enzyme having the aforementioned properties. The preferred preparation process is as follows:

(1)-(i) Assay of AATase activity

A 1 ml of a solution containing a buffer for AATase reaction (25 mM imidazole hydrochloride buffer (pH 7.5), 1 mM acetyl-CoA, 0.1% Triton X-100, 0.5% isoamyl alcohol, 1 mM dithiothreitol, 0.1 M sodium chloride, 20% glycerol: or 10 mM phosphate buffer (pH 7.5), 1 mM acetyl-CoA, 0.1% Triton X-100, 0.5% isoamyl alcohol, 1 mM dithiothreitol, 0.1 M sodium chloride, 20% glycerol) and the enzyme of the present invention was encapsulated into a 20 ml vial and reacted at 25 DEG C for 1 hour. After incubation, the vial was opened and the reaction was stopped by adding 0.6 g of sodium chloride. n-Butanol was added as an internal standard to the reaction mixture up to 50 ppm. The vial was capped with a teflon stopper.Then, the isoamyl acetate generated was determined with the head space gas chromatography (Shimadzu GC-9A, HSS-2A) under the following condition:
Column: glass column 2.1 m x 3 mm
Stationary phase: 10% Polyethylene Glycol 1540 Diasolid L (60/80 mesh)
Column temperature: 75 DEG C
Injection temperature: 150 DEG C
Carrier gas: nitrogen
Flow rate: 50 ml/min
Sample volume: 0.8 ml.

(1)-(ii) Preparation of crude enzyme

Yeast cells of KYOKAI No. 7 were inoculated in 500 ml of a YPD culture (1% yeast extract, 2% bactopeptone, 2% glucose) and cultured at 15 DEG C for 3 days. A 25 ml of the culture solution was inoculated into 1000 ml of a YPD culture medium in 20 set of Erlenmeyer flasks having a 200 ml volume and cultured at 30 DEG C for 12 hours. Cells were then collected by centrifugation (3,000 rpm, 10 min) and suspended into a buffer (50 mM Tris hydrochloride buffer (pH 7.5), 0.1 M sodium sulfite, 0.8 M potassium chloride) having a volume 10 times that of the cells. After this, "ZYMOLYASE 100T" (yeast cell cleaving enzyme commercially available from SEIKAGAKU KOGYO K.K.; Japanese Patent No. 702095, US Patent No. 3,917,510) was added in an amount of 1/1,000 to the weight of the cells. The mixture was incubated with shaking at 30 DEG C for 1 hour.Then, the resulting protoplast was collected by centrifugation at 3,000 rpm for 5 minutes, suspended in 400 ml of a buffer for the disruption of cells (25 mM imidazole hydrochloride buffer (pH 7.5), 0.6 M potassium chloride, 1 mM sodium ethylenediaminetetraacetate (EDTA)) and disrupted with a microbe cell disrupting apparatus "POLYTRON PT10" (KINEMATICA Co.). The cell debris were removed by centrifugation at 45,000 rpm to give a crude enzyme solution.

(1)-(iii) Preparation of microsome fraction

After the crude enzyme solution obtained in (1)-(ii) was centrifuged at 100,000 x G for 2 hours, and the resulting precipitate ("microsomal fraction") was suspended in 40 ml of a buffer (25 mM imidazole hydrochloride buffer (pH 7.5), 1 mM dithiothreitol). When the suspension was not immediately used, it was stored at -20 DEG C.

(1)-(iv) Preparation of solubilized enzyme

After the microsomal fraction obtained in (1)-(iii) was placed in a Erlenmeyer flask, Triton X-100 was added in an amount of 1/100 of the volume. The mixture was gently agitated with a magnetic stirrer at 4 DEG C for 60 minutes so that the mixture was not foamed. The mixture was then centrifuged at 100,000 x G for 2 hours. The supernatant was then dialyzed overnight against the buffer A (25 mM imidazole hydrochloride buffer (pH 7.2), 0.1% Triton X-100, 0.5% isoamyl alcohol, 1 mM dithiothreitol, 20% glycerol).

(1)-(v) Purification of enzyme

By repeating the procedures (1)-(ii) and (1)-(iii) twenty times, microsomal fraction was obtained and stored at -20 DEG C. Then by subjecting the procedure (1)-(iv) to the microsomal fraction, the solubilized enzyme fraction for further purification was obtained. The solubilized enzyme fraction was first applied to a POLYBUFFER EXCHANGER 94 column (Pharmacia) (adsorption: buffer A; elution: buffer A + a gradient of 0.0 to 0.6 M sodium chloride).

The active fraction was collected and repeatedly applied to the POLYBUFFER EXCHANGER 94 column.

The active fraction was further purified in the manner as shown in Table 1. That is, the active fraction was purified by
(1) ion-exchange column chromatography with DEAE Toyopearl 55 (TOSOH, adsorption: buffer A; elution: buffer A + a gradient of 0.0 to 0.2 M sodium chloride);
(2) gel filtration chromatography with Toyopearl HW60 (TOSOH) using buffer B (10 mM phosphate buffer (pH 7.5), 0.1% Triton X-100, 0.5% isoamyl alcohol, 1 mM dithiothreitol, 0.1 M sodium chloride, 20% glycerol);
(3) hydroxyapatite column chromatography (Wako Pure Chemical Industries, Ltd., adsorption: buffer B; elution: buffer B + a gradient of 10 to 50 mM phosphate buffer (pH 7.5); or
(4) octyl sepharose column chromatography (Pharmacia, adsorption: 50 mM imidazole hydrochloride (pH 7.5), 0.5% isoamyl alcohol, 1 mM dithiothreitol, 0.1 M sodium chloride, 20% glycerol; elution: 50 mM imidazole hydrochloride (pH 7.5), 0.1% Triton X-100, 0.5% isoamyl alcohol, 1 mM dithiothreitol, 0.1 M sodium chloride, 20% glycerol).

As shown in Table 1, AATase was purified approximately 2,000 times on the basis of the specific activity. However, a small amount of other proteins was still observed in SDS-PAGE with silver stain, thus indicating insufficient purification.

Thus, the present inventors have carried out affinity chromatography based on the specific affinity between 1-hexanol and AATase. Hexanol Sepharose 4B column was prepared with 6-amino-1-hexanol (Wako Pure Chemical Industries, Ltd.) and CNBr activated Sepharose 4B (Pharmacia) as a support according to the protocol by Pharmacia. Affinity chromatography was conducted with the column (adsorption: 5 mM phosphate buffer (pH 7.2), 0.1% Triton X-100, 20% glycerol, 1 mM dithiothreitol; elution: sodium chloride with a gradient from 0.0 to 0.2 M). The active fraction thus obtained as shown in Fig. 9 was subjected to SDS-PAGE and stained with silver. The AATase was successfully purified to homogenity since the active fraction was an enzyme which afforded a single band as shown in Fig. 8.

(2) Properties of AATase

(2)-(i) Substrate specificity

According to studies of substrate specificity of AATase to various kinds of alcohol by using the aforementioned analytical apparatuses and methods, AATase acts on a variety of alcohol having 1 - 5 carbon atoms. AATase acts more efficiently on alcohols having higher number of carbon atoms. In addition, AATase acts more efficiently on straight chain alcohols rather than branched chain alcohols (Fig. 9).

(2)-(ii) Optimum pH and pH stability

In order to examine the effect of pH on the stability of the enzyme, the enzyme was maintained at respective pH of from pH 5 to 9 (pH 5 - 6: 50 mM citrate-phosphate buffer; pH 6 - 8: 50 mM phosphate buffer; pH 8 - 9: 50 mM Tris-phosphate buffer) under the condition of 4 DEG C for 22 hours. The enzyme activity was assayed at pH 7.5 with 0.2M disodium phosphate according to the method (1)-(i).

In order to evaluate the effect of pH on the activity of the enzyme, the enzyme activities were assayed at respective pH of from 5 to 9 (pH 5 - 6: 50 mM citrate-phosphate buffer; pH 6 - 8: 50 mM phosphate buffer; pH 8 - 9: 50 mM Tris-phosphate buffer) according to the method (1)-(i).

The enzyme of the present invention was stable within the pH range from 7.5 to 8.5. The optimum pH was 8.0.

(2)-(iii) Optimum temperature and thermal stability

In order to examine the effect of temperature on the activity of the enzyme, the enzyme activities were assayed at various temperatures according to the method of (1)-(i).

In addition, after the enzyme incubated at each temperature for 30 minutes, the enzyme activities were assayed according to the method of (1)-(i).

The optimum temperature was 25 DEG C. The enzyme was stable at 4 DEG C, but it was very unstable at a temperature of higher than 4 DEG C.

(2)-(iv) Inhibition

For the examination of effects of various inhibitors on the enzyme activity, enzyme assay was carried out in a reaction buffer described in (1)-(i) containing inhibitors (1mM) shown in Table 2 according to the method of (1)-(i). The results are shown in Table 2. The enzyme according to the present invention is believed to be an SH enzyme, because it was inhibited strongly by p-chloromercuribenzoic acid (PCMB) and dithiobis(2-nitrobenzoic acid) (DTNB).

Id=Table 2 Columns=4
Head Col 1: Inhibitor (1 mM)
Head Col 2: Relative activity(%)
Head Col 3: Inhibitor (1 mM)
Head Col 4:Relative activity(%)
None100ZnCl212.7
KCl98.6MnCl253.3
MgCl286.2HgCl20
CaCl287.7SnCl252.0
BaCl273.7TNBS
*
16.8
FeCl354.5PCMB
*
0
CoCl237.6DTNB
*
0
CdCl23.1PMSF
*
70.2
NiCl322.31,10-phenanthroline
CuSO4087.9
*1 mM TNBS: Trinitrobenzenesulfonic acid,
0.1 mM PCMB: p-Chloromercuribenzoic acid
0.1 mM DTNB: Dithiobis(2-nitrobenzoic acid)
1 mM PMSF: Phenylmethanesulfonyl fluoride.

(2)-(v) Effects of fatty acids on enzyme activity

Various fatty acids were added in an amount of 2 mM to the reaction buffer of (1)-(i) to examine the effect of the fatty acids on the enzyme activity. The activity was assayed according to the method (1)-(i). The results are shown in Table 3.
Id=Table 3 Columns=2 Influence of fatty acids on the enzyme activity
Head Col 1: Fatty acid (2 mM)
Head Col 2: Relative activity (%)
None100
Myristic acid C14H28O260.5
Palmitic acid C16H32O288.1
Palmitoleic acid C16H30O216.7
Stearic acid C18H36O280.5
Oleic acid C18H34O259.6
Linoleic acid C18H32O24.3
Linolenic acid C18H30O232.0

(3) Sequencing of partial amino acid sequence

Partial amino acid sequence was determined according to the method described by Iwamatsu (SEIKAGAKU, 63, 139 (1991)) using a polyvinylidene difluoride (PVDF) membrane. The AATase prepared in (1)-(v) was dialyzed against 3 liter of 10 mM formic acid for 1 hour and then lyophilized. The lyophilized enzyme was suspended in a buffer for electrophoresis (10% glycerol, 2.5% SDS, 2% 2-mercaptoethanol, 62 mM Tris hydrochloride buffer (pH 6.8)) and subjected to SDS-PAGE. Then, the enzyme was electroblotted onto a PVDF membrane of 10 cm X 7 cm ("ProBlot", Applied Biosystems) using ZARTBLOT IIs model (ZARTRIUS Co.). The electroblotting was carried out at 160 mA for 1 hour according to "Pretreatment method of a sample in PROTEIN SEQUENCER (1)" edited by SHIMAZDU SEISAKUSHO.

PVDF-immobilized enzyme was then cut off and dipped into about 300 mu l of a buffer for reduction (6 M guanidine hydrochloride - 0.5 M Tris hydrochloride buffer (pH 3.5), 0.3% EDTA, 2% acetonitrile) with 1 mg of dithiothreitol (DTT) and reduced under argon at 60 DEG C for about 1 hour. A solution of 2.4 mg of monoiodoacetic acid in 10 mu l of 0.5 N sodium hydroxide was added. The mixture was then stirred in darkness for 20 minutes. After the PVDF membrane was taken out and washed sufficiently with 2% acetonitrile, the membrane was further stirred in 0.1% SDS for 5 minutes. The PVDF membrane was next rinsed lightly with water, dipped into 0.5% polyvinylpyrrolidone -40 -100 mM acetic acid and left standing for 30 minutes. The PVDF membrane was washed thoroughly with water and cut into square chips having a side of about 1 mm.The chips were dipped into a digestion buffer (8% acetonitrile, 90 mM Tris hydrochloride buffer (pH 9.0)) and digested at room temperature for 15 hours after 1 pmol of ACROMOBACTER PROTEASE I (Wako Pure Chemical Industries, Ltd.) was added. The digested products was separated by reverse phase high performance liquid chromatography (model L6200, HITACHI) with a C8 column (NIPPON MILIPORE, LTD; mu -Bondasphere 5C8, 300A, 2.1 X 150 mm) to give a dozen or so peptide fragments. The elution of the peptide was carried using the solvent A (0.05% trifluoroacetic

acid) with a linear gradient from 2 to 50% of the solvent B (2-propanol/ acetonitrile (7:3) containing 0 .02% trifluoroacetic acid) at a flow rate of 0.25 ml/min. The amino acid sequencing of the peptide fragments thus was conducted by the automatic Edman degradation method with a vapor phase protein sequencer model 470 (Applied Biosystems) according to manufacturer's instructions.

As a result, the following amino acid sequences were determined:
peak 1 Lys Trp Lys
peak 2 Lys Tyr Val Asn Ile Asp
peak 3 Lys Asn Gln Ala Pro Val Gln Gln Glu Cys Leu
peak 4 Lys Gly Met Asn Ile Val Val Ala Ser
peak 5 Lys Tyr Glu Glu Asp Tyr Gln Leu Leu Arg Lys
peak 6 Lys Gln Ile Leu Glu Glu Phe Lys
Peak 7 Lys Leu Asp Tyr Ile Phe Lys
Peak 8 Lys Val Met Cys Asp Arg Ala Ile Gly Lys

(4) Cloning of DNA encoding AATase from sake yeast

(i) Preparation of sake yeast library

Yeast cells of KYOKAI No. 7 were grown in 1 liter of a YPD medium up to O.D.600 = 10, collected and washed with sterilized water. The cells were suspended in SCE solution (1 M sorbitol, 0.125 M EDTA, 0.1 M trisodium citrate (pH 7), 0.75% 2-mercaptoethanol, 0.01% "ZYMOLYACE 100T" (SEIKAGAKU KOGYO K.K.) in a ratio of 2 ml of SCE solution per 1g of the cells, incubated at 37 DEG C for about 2 hours and protoplastized completely. The resulting protoplast was suspended in Lysis Buffer (0.5 M Tris hydrochloride buffer (pH 9), 0.2 M EDTA, 3% sodium dodecyl sulfate (SDS)) in an ratio of 3.5 ml of the buffer per 1g of the cells. The mixture was then stirred gently at 65 DEG C for 15 minutes to lyse the cells completely.After the lysis, the mixture was cooled to room temperature, a 10 ml of the mixture was cautiously placed on each of 23.5 ml of 10% - 40% sucrose density gradient solution (0.8 M sodium chloride, 0.02 M Tris hydrochloride buffer (pH 8), 0.01 M EDTA, 10% - 40% sucrose) which had been previously prepared in HITACHI ultracentrifugation tubes 40PA. It was centrifuged with a HITACHI ULTRACENTRIFUGE SCP85H at 4 DEG C and 26,000 rpm for 3 hours. After the centrifugation, the resulted solution was recovered with a graduated pippete (komagome) in an amount of about 5 ml from the bottom of the tube. The DNA sample thus recovered was dialyzed overnight against 1 liter of a TE solution.

The chromosomal DNA thus obtained was partially digested with Sau3AI according to the method by Frischauf et al. (Methods in Enzymology, 152, 183, Academic Press, 1987), placed again on 10% - 40% sucrose density gradient solution and centrifuged at 20 DEG C and 25,000 rpm for 22 hours. After centrifugation, the ultracentrifugation tube was pierced at the bottom with a needle, and 0.5 ml of the density gradient solution was fractionated in every sampling tube. A portion of each fraction was subjected to agarose gel electrophoresis to confirm the molecular weight of the chromosomal DNA. Then, the 15 - 20 kb DNA was collected and recovered by ethanol precipitation.

The digested chromosomal DNA (1 mu g) and the lambda -EMBL3 vector (1 mu g) of a lambda -EMBL3/BamHI vector kit (manufactured by STPATAGENE, purchased from FUNAKOSHI) were ligated at 16 DEG C overnight. The ligation product was packaged using a GIGAPACK GOLD (manufactured by STRATAGENE, purchased from FUNAKOSHI). The ligation and packaging were conducted according to manufacturer's instructions.

The host strain P2392 of the lambda -EMBL3 vector kit was infected with a 50 mu l of the packaged solution. One inoculation loop amount of P2392 was cultured in 5 ml of a TB culture medium (1% bactotriptone (DIFCO), 0.5% sodium chloride, 0.2% maltose, pH 7.4) at 37 DEG C overnight. Then, 1 ml of the culture was inoculated into 50 ml of a TB culture medium and cells were grown up to O.D. 600 = 0.5. After the culture fluid was cooled on an ice bath, the cells were collected by centrifugation and suspended in 15 ml of an ice-cooled 10 mM magnesium sulfate solution. To 1 ml of the cells were added 0.95 ml of an SM solution (0.1 M sodium chloride, 10 mM magnesium sulfate, 50 mM Tris hydrochloride buffer (pH 7.5), 0.01% gelatin) and 50 mu l of the packaging solution. The mixture was slightly stirred and kept at a temperature of 37 DEG C for 15 minutes.A 200 mu l portion of the mixture was added into 7 ml of a BBL soft agar culture medium (1% Tripticase peptone (BBL), 0.5% sodium chloride, 0.5% agarose (Sigma)) which had been maintained at a temperature of 47 DEG C. The mixture was slightly mixed and overlaid for spreading on a BBL agar plate (1% Tripticase peptone, 0.5% sodium chloride, 1.5% Bactoagar (DIFCO)) having a diameter of 15 cm.

The overlaid plate was incubated at a temperature of 37 DEG C for 8 hours. A pharge library which contains approximately 30,000 clones having yeast chromosmal DNA fragments, on 10 overlaid agar plates were thus obtained.

The library was transferred to a nylon membrane for cloning. A hybridization transfer membrane (NEN) having a diameter of 15 cm was contacted with the overlaid agar plate for about 2 minutes to prepare two sets of the membranes on which the phages were transferred and 20 sheets in total. The membranes were placed with the surface which had been contacted with the agar plate up on a filter paper impregnated with an alkali denaturating solution (1.5 M sodium chloride, 0.5 N sodium hydroxide) and left standing for about 5 minutes. The membranes were then displaced on a filter paper impregnated with a neutralizing solution (3M sodium acetate (pH 5.8)), left standing for about 5 minutes, then dried at room temperature and further dried in vacuum at 80 DEG C for 1 hour.The agar plate from which the library had been transferred were stored at 4 DEG C.

(ii) Synthesis and Labelling of probes

The following synthetic probes were prepared using a DNA synthesizer "Model 380B" (manufactured by APPLIED BIOSYSTEMS) on the basis of the partial amino acid sequence of Peak 5 and Peak 2 obtained in (3): All of the synthesis reagents such as phosphoamidite were purchased from APPLIED BIOSYSTEMS and were used according to manufacturer's instructions.

The synthetic DNA thus obtained was treated with 3 ml of an 28% aqueous ammonia at 60 DEG C for 4 hours and then purified with an Oligonucleotide Purification Cartriges manufactured by APPLIED BIOSYSTEMS.

The two synthetic probes were individually labelled with [gamma -P]ATP (ca. 6000 Ci/mM). Each probe DNA (ca. 250 ng) was subjected to reaction in 200 mu l of a reaction solution containing 10 units of T4 polynucleotide kinase, 500 mu Ci of [gamma -P]ATP and a phosphate buffer (0.1 mM spermidine, 0.1 mM EDTA, 10 mM magnesium chloride, 5 mM DTT, 50 mM Tris hydrochloride (pH 7.6)) at 37 DEG C for 1 hour, and kept at a temperature of 70 DEG C for 10 minutes. Unincorporated [gamma -P]ATP was removed by the purification with a DE52 manufactured by WATTMAN.

(iii) Cloning by plaque hybridization

The cloning by plaque hybridization was carried out by first, second and third screenings as follows:

In the first screening, 20 sheets of the membrane on which the yeast library prepared in (4)-(i) had been transferred were dipped into 200 ml of a hybridization solution (6 x SSPE (1.08 M sodium chloride, 0.06 M sodium phosphate, 6 mM EDTA, pH 7.4), 5 x a Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 0.5% SDS, 10 mu g/ml single strand salmon sperm DNA) and incubated for prehybridization at 60 DEG C for 3 hours.

The [gamma -P]ATP labelled probe 5 prepared in (4)-(ii) was kept at 95 DEG C for 5 minutes and cooled with ice-water. The twenty sheets of the prehybrized membrane were dipped into a mixed solution of the denatured probe 5 and 400 ml of a hybridization solution and incubated gently at 30 DEG C overnight to hybridize the membrane with the labelled probe 5.

The hybridization solution was discarded. In order to remove the excessive probe 5 from the membrane, the membrane was shaken gently in 400 ml of 2 X SSC (0.3 M sodium chloride, 0.03 M sodium citrate) at 30 DEG C for 20 minutes. The membrane was then contacted with a X-ray film and exposed at -80 DEG C overnight. As positive clones 49 plaques which had sensitized both of the two sheets were subjected to the second screening.

In the second screening, these plaques on the original agar plates were picked with an aseptic Pasteur's pipette and suspended into 1 ml of SM. After A 1/1000 dilution of the suspension was prepared, 100 mu l of the P2392 microbial solution was infected with a 100 mu l portion of the dilution in the same manner as in the preparation of the library, mixed with 3 ml of a BBL soft agar medium and overlaid on a BBL agar plates having a diameter of 9 cm. After plaques had appeared, 49 sets of two membrane sheets to one clone were prepared in the same manner as described in (3)-(iii). The same procedure as in the first screening was repeated with the [gamma -P]ATP labelled probe 2 which had been prepared in (4)-(ii). Fifteen plaques as the positive clones were subjected to the third screening.

In the third screening, using the [gamma -P]ATP labelled probe 5, the same procedure as in the second screening was repeated. Finally, 14 positive clones were obtained.

An overnight culture of E.coli P2392 in TB medium was concentrated four times in TB medium containing 10 mM MgSO4. Then 20 mu l of each positive clone which had been prepared in a concentration of 10 to 10 plaque/ml was infected to 5 ml of this cell suspension. This infected all suspension was kept at 37 DEG C for 15 minutes, then inoculated into 50 ml of TB medium containg 10 mM MgSO4 and cultured for 6 hours with shaking. Then, CCl4 was added to the cell culture and the culture was incubated with shaking at 37 DEG C for 30 minutes to lyse P2392 and entrifuged at 10,000 rpm for 10 minutes to recover the supernatant. DNase (TAKARA SHUZO) and RNase (BERINGER-MANNHEIM) were added to the supernatant up to 10 mu g/ml, respectively. The mixture was then kept at 37 DEG C for 30 minutes.After the polyethylene glycol solution (20% Polyethylene Glycol 6000, 2.5 M sodium chloride) was added in an amount of 30 ml, the mixture was left standing at 4 DEG C overnight. Centrifugation was conducted at 10,000 rpm for 10 minutes. After the supernatant was discarded, the precipitate was suspended in 3 ml of SM. EDTA (pH 7.5) and SDS were added to the suspension up to 20 mM and 0.1%, respectively. The mixture was kept then at 55 DEG C for 4 minutes followed by adding the phenol solution (phenol (25): chloroform (24): isoamyl alcohol (1)). The mixture was slowly stirred for 10 minutes, centrifuged 10,000 rpm for 10 minutes to recover the DNA layer (aqueous layer). After this procedure was repeated again, 0.33 ml of 3M sodium acetate and 7.5 ml of ethanol were added to the aqueous layer, and the mixture was stirred and left standing at -80 DEG C for 30 minutes.After the mixture was centrifuged at 10,000 rpm for 10 minutes, the precipitate was rinsed with 70% ethanol, then remove 70% ethanol, and the precipitate was dried up and dissolved in 500 mu l of TE. Each of the phage DNAs thus obtained was cut with a variety of restriction enzymes and compared with each other by electrophoresis. Although the fourteen positive clones appeared consist of not only those containing the whole of the DNA sequence capable of producing AATase but those having partial deletions, all of the clones were those which cloned the identical site on the yeast chromosome. The restriction map of 6.6 kb Xbal fragment containing the whole length of the DNA sequence among these clones are shown in Fig. 3. The DNA sequencing was carried out according to the dideoxy method with a Xbal fragment which had been subcloned

in pUC119 (TAKARA SHUZO).The DNA sequence of the gene encoding AATase is shown in Fig. 1.

(5) Preparation of DNA encoding AATase from brewery lager yeast

Using the sake yeast AATase gene as a probe, a DNA strands hybridized with the sake yeast (KYOKAI No. 7) AATase gene were cloned from brewery lager yeast. The 1.6 kb HindIII (the range within the arrow) fragment shown in Fig. 3 (50 ng) was reacted with 100 mu Ci of [alpha P]dCTP (ca. 3,000 Ci/mM) using a Multiprime Labelling Kit (AMERSHAM JAPAN K.K.). Cloning by plaque hybridization was performed with this reaction product as a probe and the brewery lager yeast library containing 30,000 phage clones prepared in the same manner as described in (4)-(i). Hybridization temperature was set at 50 DEG C. The membranes were gently incubated at 50 DEG C in 2 x SSC for 30 minutes and in 0.2 x SSC (0.03 M sodium chloride, 3 mM sodium citrate) for 30 minutes in order to remove the excessive probes. In the first screening, 60 positive clones were obtained. These positive plaques were subjected to the second screening in the same manner as described in (4)-(iii). Hybridization was repeated with the same probe under the same condition as described above to give 30 positive clones. DNA was extracted from these positive clones and subjected to restriction analysis. The results shows that those positive clones are two groups. The restriction maps of the insert DNA of these two groups are quite different, thus it has been suggested these insert DNAs present on different locus of yeast chromosome. Fig. 6 show the restriction maps of the DNA fragment containing AATase 1 and 2. These clones are referred to hereinafter as "brewery yeast AATase 1 gene" and "brewery yeast AATase 2 gene", respectively.

The DNA sequences of the brewery yeast AATase 1 gene and the brewery yeast AATase 2 gene were determined in the same manner as described in (4)-(iii). The DNA sequences of the brewery yeast AATase 1 gene and the brewery yeast AATase 2 gene are shown in Figs. 2 and 17, respectively. The AATase 2 gene was a DNA fragment which produces a polypeptide having an AATase activity in either case of the DNA sequence from A to C or the DNA sequence from B to C.

(6) Preparation of a vector containing an AATase gene and cultivation of a yeast transformed by the vector

(i) Construction of an expression vector for Saccharomyces cerevisiae

A 6.6 kb XbaI fragment (AAT-K7) of the sake yeast AATase gene obtained in (4)-(iii) and shown in Fig. 3 was prepared. The fragment was cloned into the NheI site of the yeast vector YEp13K containing the replication origin of the yeast 2 mu m DNA and the yeast LEU2 gene as a marker to construct the expression vector YATK11 (Fig. 11).

In the same manner, a 6.6 kb XbaI fragment (AAT-1) of the brewery yeast AATase gene 1 obtained in (5) and shown in Fig. 4 was cloned into the NheI site of YEp13K to construct the expression vector YATL1 (Fig. 12).

In addition, a 5.6 kb BglII fragment (AAT-2) of the brewery yeast AATase gene 2 shown in Fig. 4 was cloned into the BamHI site of YEp13K to construct the expression vector YATL2 (Fig. 13).

(ii) Construction of an expression vector for sake yeast KYOKAI No. 9

Plasmid pUC4k (Pharmacia) containing a G418 resistant gene was cut with SalI. Then, the resulting fragment containing the G418 resistant gene was cloned into the SalI site of the YATK11 to construct a vector YATK11G for transfecting the AATase gene into sake yeast (Fig. 14).

(iii) Construction of an expression vector for brewery lager yeast

(iii-a) Preparation of G418 resistant marker

The 2.9 kb HindIII fragment containing PGK gene (Japanese Patent Laid-Open Publication No. 26548/1990) was cloned into pUC18 (TAKARA SHUZO). Plasmid pUCPGK21 containing a PGK promoter and a terminator was shown in Fig. 16.

G418 resistant gene was cloned from the plasmid pNEO (Pharmacia) into the pUCPGK21 by the process described in Fig. 16 to construct pPGKneo2.

(iii-b) Construction of expression vectors

pPGKNEO2 was digested with SalI to generate the ca. 2.8 kb fragment containing the PGK promoter, the G418 resistant gene and the PGK terminator. This fragment was then cloned into the XhoI site of YATL1 to construct

YATL1G (Fig. 15).

(7) Transformation of yeasts with AATase gene

In order to confirm that the cloned AATase genes in (4)-(iii) and (5) Produces AATase, yeast cells were transformed with these vectors prepared in (6), and AATase activity of the transformants were measured.

The transfection of the plasmid into Saccharomyces cereviciae TD4 (a, his, leu, ura, trp) was carried out according to the lithium acetate method (J. Bacteriol., 153, 163 (1983)) to give YATK11/TD4, YATL1/TD4 and YATL2/TD4 (SKB105 strain).

The transformant of SAKE YEAST KYOKAI NO. 9 (SKB106 strain) was obtained according to the following procedure. The strain, in to which the plasmid had been transfected by the lithium acetate method, was spread onto YPD agar plates containing G418 (300 mu l/ml). The plates were incubated at 30 DEG C for 3 days. Colonies grown up were inoculated again in a YPD agar medium containing G418 (500 mu g/ml) and cultured at 30 DEG C for 2 days to give the transformants.

YATL1G was transfected into the strain 2155 of the brewery lager yeast Alfred Jorgensen Laboratory (Denmark) (AJL2155 strain) in the following procedure. The yeast was cultured with shaking in 100 ml of a YPD medium at 30 DEG C until O.D.600 = 16. Cells were collected, rinsed once with sterilized water, then rinsed once with 135 mM Tris buffer (pH 8.0) and suspended in the same buffer so that the suspension had a microbial concentration of 2 x 10 cells/ml. To 300 mu l of the suspension were added 10 mu g of YATL1G, 20 mu g of calf thymus DNA (Sigma) as a carrier DNA and finally 1200 mu l of 35% PEG4000 (which had been subjected to sterilized filtration). The mixture was then stirred sufficiently. A 750 mu l portion of the stirred fluid was poured into a cuvette for Gene Pulser (BIORAD) and subjected once to an electric pulse treatment under the conditions of 1 mu F and 1000 v.The cell suspension was transferred from the cuvette to a 15 ml tube and left standing at 30 DEG C for 1 hour. The cells were collected by centrifugation at 3,000 rpm for 5 minutes, suspended in 1 ml of a YPD medium and incubated at 30 DEG C for 4 hour. The cells were collected, suspended in 600 mu l of sterilized water. A 150 mu l of the suspension were spread onto YPD agar plates containing G418 (100 mu g/ml). The plates were incubated at 30 DEG C for 3 days to obtain the transformant SKB108.

The AATase activities of the transformant into which the AATase gene had been transfected and the control strains were measured. An SD liquid medium containing a leucine-free mixed amino acid solution (0.65% yeast nitrogen base (amino acid free; DIFCO), 2% glucose) was used for cultivating transformants of Saccharomyces cerevisiae TD4; an YPD liquid medium containing G418 (400 mu g/ml) was used for cultivating transformants of sake yeast KYOKAI No. 9,; a YPD medium containing G418 (10 mu g/ml) was used for cultivating transformants of brewery lager yeast AJL2155 strain. A 25 ml portion of the shaking culture product at 30 DEG C for about 16 hours was added to 1000 ml of the culture medium and the culture was incubated at 30 DEG c for 12 to 18 hours under static conditions.

The preparation of a crude enzyme and the assay of its activity were performed according to the procedures described in (1)-(ii) and (1)-(i). Protein concentration was determined with a BIORAD PROTEIN ASSAY KIT (BIORAD) according to the instructions of its manual.

The results for the Saccharomyces cerevisiae TD4, the sake yeast KYOKAI No. 9 and the beer yeast AJL2155 are shown in Tables 4, 5 and 6, respectively. The results shows that the transformants of the present invention have AATase activities of 2 to 15 time higher than that of the untransformed stain.This indicates that the AATase gene according to the present invention facilely provides a strain which produces a large amount of an acetate ester such as isoamyl acetate.
Id=Table 4 Columns=2
Head Col 1: Transformants
Head Col 2: Crude enzyme activity (ppm/mg protein)
YEp13K/TD47.8
YATK11/TD484.0
YATL1/TD4116.2
YATL2/TD4 (SKB105)50.6

Id=Table 5 Columns=2
Head Col 1: Transformants
Head Col 2: Crude enzyme activity (ppm/mg protein)
K93.4
YATK11G/K9 (SKB106)11.6

Id=Table 6 Columns=2
Head Col 1: Transformants
Head Col 2: Crude enzyme activity (ppm/mg protein)
AJL21554.1
YATK11G/AJL2155 (SKB108)11.6

(8) Fermentation test of the transformants

Sake and beer were prepared by use of the yeast transformed with the AATase gene in the above (7).

(8)-(i) Production of sake with the transformant yeast

Small scale sake brewing test was carried out with 300g rice according to the feed program as shown in Table 7. Thirty grams of malted rice (koji rice) and 110 ml of water including yeast (2 x 10 cells/ml) (Koji rice) and lactic acid (0.35%(v/v)) were mixed and incubated at 15 DEG C. On the second day, 35g of steamed rice was added as the 1st feed. On the fourth day, the 2nd feed was carried out. After fermentation for 15 days, the fermentation product was centrifuged at 8,000 rpm for 30 minutes. Esters concentration of the "sake" liquor was measured. The results are shown in Table 8.The liquor produced by the transformant of the present invention has an aromatic flavor due to an enhanced amount of acetate esters such as ethyl acetate, isoamyl acetate in comparison with the liquor produced by yeast cells of KYOKAI-K9.
Id=Table 7 Columns=5 Feed program for small scale sake brewing
Head Col 1:
Head Col 2: Seed Mash
Head Col 3: 1st
Head Col 4: 2nd
Head Col 5: Total
Steamed rice35 g213 g248 g
Koji rice30 g22 g52 g
Water110 ml310 ml420 ml

(8)-(ii) Preparation of beer with transformant yeast

After yeast was added to the wort in which the original extract content was adjusted to 11 DEG P, the mixture was incubated at 8 DEG C for 8 days, centrifuged at 3,000 rpm for 10 minutes and sterilized by filtration. Esters contained in the filtrated solution was measured. The results are shown in Table 9.The transformant of the present invention produced a liquor having an enhanced amount of acetate esters such as ethyl acetate, isoamyl acetate in comarison with the liquor produced by the untransformed yeast AJL2155.
Id=Table 9 Columns=5
Head Col 1: Strain
Head Col 2: Apparent extract content (DEG P)
Head Col 3: Ethyl acetate (ppm)
Head Col 4: Isoamyl acetate (ppm)
Head Col 5: Isoamyl alcohol (ppm)
SKB108(YATL1G)2.322.80.9951.2
AJL2155(Control)2.85.90.1340.0

(9) Preparation of DNA encoding AATase from the wine yeast

The primers A and B which have homology to two different sites in the sake yeast AATase gene shown in Fig. 6 were synthesized. Polymerase chain reaction (PCR) was performed with Gene Amp Reagent Kit (TAKARA SHUZO) and DNA Thermal Cycler (Parkin-Elmer-Theters Instruments Co.) using chromosomal DNA of wine yeast as a template with the two primers to give a 1.17kb DNA fragment from the position of the primer A to the position of primer B. The process consisted of 30 cycles with annealing at 50 DEG C for 2 minutes. The reaction mixture was applied to agarose electrophoresis. The 1.17kb DNA fragment was purified from the gel, labelled with 100 mu Ci [P]dCTP using Nick Translation Kit (TAKARA SHUZO) and hybridized with 20,000 genome libraries of a wine yeast W-3 (YAMANASHI KOGYO GIJUTSU CENTER) prepared in the same manner as the sake yeast library.After the hybridization was carried out at 65 DEG C, the membranes were rinsed with 2 x SSC (1 x SSC is 15mM NaCl plus 1.5mM sodium citrate) for 20 minutes, 2 x SSC for 10 minutes and finally 0.1 x SSC with gentle shaking at 65 DEG C. As positive, 14 plaques were first obtained. Upon hybridizing these plaques with the 1.7kb fragment in the same manner as the above, 7 positive plaques having a strong hybridization signal were obtained. The phage DNAs of these positive plaques were purified and subjected to restriction enzyme analysis. As a result, it was found that all of the 7 clones were of the same DNA having the restriction map shown in Fig. 5.

Deposition of the microorganisms

The microorganisms shown below related to the present invention have been deposited at Fermentation Research Institute of Agency of Industrial Science and Technology, Japan under the following deposition numbers under the Budapest Treaty on the international Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure.
(1) SKB105 FERM BP-3828
(2) SKB106 FERM BP-3829
(3) SKB108 FERM BP-3830 YATL2, YATK11G and YATL1G can be obtained by culturing SKB105, SKB106 and SKB108, respectively, under a certain condition, extracting therefrom the total DNA of the yeast (Methods in yeast genetics, Cold Spring Harbor Laboratory, 1988), transforming Escherichia coli with this total DNA and finally

extracting the plasmids by the alkali method (lit: Molecular clonign, Cold Spring Harbor Laboratory, 1989).

A DNA fragment containing a part of the DNA sequence from A to B of the DNA sequence shown in Fig. 1 can be obtained by digesting YATK11G with an appropriate restriction enzyme. An example of a suitable DNA sequences is 1.6 kb HindIII fragment which is indicated by a double-headed arrow in Fig. 3.

Claims

1. An alcohol acetyltransferase (AATase) originated from yeast having an ability for transferring the acetyl group from acetyl-CoA to alcohol to produce an acetate ester and having a molecular weight of about 60,000 by SDS-PAGE.

2. An AATase comprising a polypeptide selected from a group consisting of:
(1a) a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;
(1b) a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 2; and
(1c) a polypeptide having an amino acid sequence from A to C or B to C of the amino acid sequence shown in Fig. 17.

3. An AATase according to claim 2, wherein the AATase is the polypeptide (1a).

4. An AATase according to claim 2, wherein the AATase is the polypeptide (1c).

5.An AATase according to claim 2, wherein the AATase is the polypeptide (1c).

6. An AATase gene comprising a DNA sequence selected from a group consisting of:'
(2a) a DNA sequence encoding a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;
(2b) a DNA sequence encoding a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 2; and
(2c) a DNA sequence encoding a polypeptide having an amino acid sequence from A to C or B to C of the amino acid sequence shown in Fig. 17.

7. An AATase gene according to claim 6, wherein the DNA sequence is the DNA sequence (2a).

8. An AATase gene according to claim 6, wherein the DNA sequence is the DNA sequence (2b).

9. An AATase gene according to claim 6, wherein the DNA sequence is the DNA sequence (2c).

10.A DNA sequence comprising an AATase gene selected from a group consisting of:'
(3a) an AATase gene comprising a DNA sequence form A to B of the DNA sequence shown in Fig. 1;
' (3b) an AATase gene comprising a DNA sequence from A to B of the DNA sequence shown in Fig. 2;
(3c) an AATase gene comprising a DNA sequence form A to C or B to C of the DNA sequence shown in Fig. 17; and
(3d) a DNA sequence capable of hybridizing with any one of genes (3a) to (3c).

11. A DNA sequence according to claim 10, wherein the DNA sequence is the AATase gene (3a).

12. A DNA sequence according to claim 10, wherein the DNA sequence is the AATase gene (3b).

13. A DNA sequence according to claim 10, wherein the DNA sequence is the AATase gene (3c).

14. An expression vector comprising an AATase gene or a DNA sequence according to claim 6 or 10.

15.A yeast transformed with an AATase gene or a DNA sequence according to claim 6 or 10.

16. A transformed yeast according to claim 15, wherein the yeast to be transformed is for producing alcoholic beverages.

17. A transformed yeast according to claim 15, transformed with an expression vector according to claim 14.

18. A process for producing an alcoholic beverage having an enriched ester flavor, comprising the step of fermenting saccharide by a yeast according to claim 15.

19. A process for isolating a DNA sequence encoding AATase, comprising the steps of:
(a) Preparing a DNA fragment having a length of at least 20 bases of a DNA sequence which encodes a polypeptide having an amino acid sequence from A to B of the amino acid sequence shown in Fig. 1;'
(b) Preparing a gene library which has been made from DNA strands having a substantially same length in the range from 5 x 10 to 30 x 10 bases obtained by cutting the chromosome of a yeast;
(c) Cloning a DNA fragment by hybridization from gene library of (b) using the DNA fragment of (a) as a probe.

```
1    AG CGT GTG AGG ACT ACT CAT TGG CTT GCG ATT TAC GGT TTT TAT ATT      47

48   TTT TGC CGC ACA TCA TTT TTT GGC CTG GTA TTG TCA TCG CGT TGA GCG     95

96   GAC TCT GAA TAT AAT CCT ATT GTT TTT TAT GGA TCT CTG GAA GCG TCT     143

144  TTT TGA AGC CAA CCC AAC AAA AAT TCG AGA CAA GAA AAT AAA AAA CGG     191
                                                        A
                                                        ↓
192  CAC TTC ATC AGT ATC ACA AAT ACC ATC AAT TTA TCA GCT CTC ATG AAT     239
                                                        Met Asn          2

240  GAA ATC GAT GAG AAA AAT CAG GCC CCC GTG CAA CAA GAA TGC CTG AAA     287
  3  Glu Ile Asp Glu Lys Asn Gln Ala Pro Val Gln Gln Glu Cys Leu Lys     18

288  GAG ATG ATT CAG AAT GGG CAT GCT CGG CGT ATG GGA TCT GTT GAA GAT     335
 19  Glu Met Ile Gln Asn Gly His Ala Arg Arg Met Gly Ser Val Glu Asp     34

336  CTG TAT GTT GCT CTC AAC AGA CAA AAC TTA TAT CGG AAC TTC TGC ACA     383
 35  Leu Tyr Val Ala Leu Asn Arg Gln Asn Leu Tyr Arg Asn Phe Cys Thr     50

384  TAT GGA GAA TTG AGT GAT TAC TGT ACT AGG GAT CAG CTC ACA TTA GCT     431
 51  Tyr Gly Glu Leu Ser Asp Tyr Cys Thr Arg Asp Gln Leu Thr Leu Ala     66

432  TTG AGG GAA ATC TGC CTG AAA AAT CCA ACT CTT TTA CAT ATT GTT CTA     479
 67  Leu Arg Glu Ile Cys Leu Lys Asn Pro Thr Leu Leu His Ile Val Leu     82

480  CCA ATA AGA TGG CCA AAT CAT GAA AAT TAT TAT CGC AGT TCC GAA TAC     527
 83  Pro Ile Arg Trp Pro Asn His Glu Asn Tyr Tyr Arg Ser Ser Glu Tyr     98

528  TAT TCA CGG CCA CAT CCA GTG CAT GAT TAT ATT TCA GTA TTA CAG GAA     575
 99  Tyr Ser Arg Pro His Pro Val His Asp Tyr Ile Ser Val Leu Gln Glu    114

576  TTG AAA CTG AGT GGT GTG GTT CTC AAT GAA CAA CCT GAG TAC AGT GCA     623
115  Leu Lys Leu Ser Gly Val Val Leu Asn Glu Gln Pro Glu Tyr Ser Ala    130

624  GTA ATG AAG CAA ATA TTA GAA GAA TTC AAA AAT AGT AAG GGT TCC TAT     671
131  Val Met Lys Gln Ile Leu Glu Glu Phe Lys Asn Ser Lys Gly Ser Tyr    146

672  ACT GCA AAA ATT TTT AAA CTT ACT ACC ACT TTG ACT ATT CCT TAC TTT     719
147  Thr Ala Lys Ile Phe Lys Leu Thr Thr Thr Leu Thr Ile Pro Tyr Phe    162

720  GGA CCA ACA GGA CCG AGT TGG CGG CTA ATT TGT CTT CCA GAA GAG CAC     767
163  Gly Pro Thr Gly Pro Ser Trp Arg Leu Ile Cys Leu Pro Glu Glu His    178

768  ACA GAA AAG TGG AAA AAA TTT ATC TTT GTA TCT AAT CAT TGC ATG TCT     815
179  Thr Glu Lys Trp Lys Lys Phe Ile Phe Val Ser Asn His Cys Met Ser    194

816  GAT GGT CGG TCT TCG ATC CAC TTT TTT CAT GAT TTA AGA GAC GAA TTA     863
195  Asp Gly Arg Ser Ser Ile His Phe Phe His Asp Leu Arg Asp Glu Leu    210

864  AAT AAT ATT AAA ACT CCA CCA AAA AAA TTA GAT TAC ATT TTC AAG TAC     911
211  Asn Asn Ile Lys Thr Pro Pro Lys Lys Leu Asp Tyr Ile Phe Lys Tyr    226

912  GAG GAG GAT TAC CAA TTG TTG AGG AAA CTT CCA GAA CCG ATC GAA AAG     959
227  Glu Glu Asp Tyr Gln Leu Leu Arg Lys Leu Pro Glu Pro Ile Glu Lys    242

960  GTG ATA GAC TTT AGA CCA CCG TAC TTG TTT ATT CCG AAG TCA CTT CTT    1007
243  Val Ile Asp Phe Arg Pro Pro Tyr Leu Phe Ile Pro Lys Ser Leu Leu    258
```

FIG. 1 (a)

```
1008  TCG GGT TTC ATC TAC AAT CAT TTG AGA TTT TCT TCA AAA GGT GTC TGT   1055
 259  Ser Gly Phe Ile Tyr Asn His Leu Arg Phe Ser Ser Lys Gly Val Cys    274

1056  ATG AGA ATG GAT GAT GTG GAA AAA ACC GAT GAT GTT GTC ACC GAG ATC   1103
 275  Met Arg Met Asp Asp Val Glu Lys Thr Asp Asp Val Val Thr Glu Ile    290

1104  ATC AAT ATT TCA CCA ACA GAA TTT CAA GCG ATT AAA GCA AAT ATT AAA   1151
 291  Ile Asn Ile Ser Pro Thr Glu Phe Gln Ala Ile Lys Ala Asn Ile Lys    306

1152  TCA AAT ATC CAA GGT AAG TGT ACT ATC ACT CCG TTT TTA CAT GTT TGT   1199
 307  Ser Asn Ile Gln Gly Lys Cys Thr Ile Thr Pro Phe Leu His Val Cys    322

1200  TGG TTT GTA TCT CTT CAT AAA TGG GGT AAA TTT TTC AAA CCA TTG AAC   1247
 323  Trp Phe Val Ser Leu His Lys Trp Gly Lys Phe Phe Lys Pro Leu Asn    338

1248  TTC GAA TGG CTT ACG GAT ATT TTT ATC CCC GCA GAT TGC CGC TCA CAA   1295
 339  Phe Glu Trp Leu Thr Asp Ile Phe Ile Pro Ala Asp Cys Arg Ser Gln    354

1296  CTA CCA GAT GAT GAT GAA ATG AGA CAG ATG TAC AGA TAT GGC GCT AAC   1343
 355  Leu Pro Asp Asp Asp Glu Met Arg Gln Met Tyr Arg Tyr Gly Ala Asn    370

1344  GTT GGA TTT ATT GAC TTC ACC CCA TGG ATA AGC GAA TTT GAC ATG AAT   1391
 371  Val Gly Phe Ile Asp Phe Thr Pro Trp Ile Ser Glu Phe Asp Met Asn    386

1392  GAT AAC AAA GAA AAA TTT TGG CCA CTT ATT GAG CAC TAC CAT GAA GTA   1439
 387  Asp Asn Lys Glu Lys Phe Trp Pro Leu Ile Glu His Tyr His Glu Val    402

1440  ATT TCG GAA GCT TTA AGA AAT AAA AAG CAC CTC CAT GGC TTA GGG TTC   1487
 403  Ile Ser Glu Ala Leu Arg Asn Lys Lys His Leu His Gly Leu Gly Phe    418

1488  AAT ATA CAA GGC TTC GTT CAA AAA TAT GTG AAT ATT GAC AAG GTA ATG   1535
 419  Asn Ile Gln Gly Phe Val Gln Lys Tyr Val Asn Ile Asp Lys Val Met    434

1536  TGC GAT CGT GCC ATC GGG AAA AGA CGC GGA GGT ACA TTG TTA AGC AAT   1583
 435  Cys Asp Arg Ala Ile Gly Lys Arg Arg Gly Gly Thr Leu Leu Ser Asn    450

1584  GTA GGT CTG TTT AAT CAG TTA GAG GAG CCC GAT GCC AAA TAT TCT ATA   1631
 451  Val Gly Leu Phe Asn Gln Leu Glu Glu Pro Asp Ala Lys Tyr Ser Ile    466

1632  TGC GAT TTG GCA TTT GGC CAA TTT CAA GGA TCC TGG CAC CAA GCA TTT   1679
 467  Cys Asp Leu Ala Phe Gly Gln Phe Gln Gly Ser Trp His Gln Ala Phe    482

1680  TCC TTG GGT GTT TGT TCG ACT AAT GTA AAG GGG ATG AAT ATT GTT GTT   1727
 483  Ser Leu Gly Val Cys Ser Thr Asn Val Lys Gly Met Asn Ile Val Val    498

1728  GCT TCA ACA AAA AAT GTT GTT GGT AGC CAA GAA TCT CTC GAA GAG CTT   1775
 499  Ala Ser Thr Lys Asn Val Val Gly Ser Gln Glu Ser Leu Glu Glu Leu    514
                                                 B
                                                 ↓
1776  TGC TCC ATT TAT AAA GCT CTC CTT TTA GGC CCT TAG ATC TCA CAT GAT   1823
 515  Cys Ser Ile Tyr Lys Ala Leu Leu Leu Gly Pro ***

1824  GCT TGA CTG ATA TTA TTC GAC AAT ATG ATT ATG TCG TGT AAA TAA CCC   1871


1872  ACT TTC ATG TTG TCA CTC CCT CGG CTT TGG TTG GTT AAA GGG ACT TAT   1919


1920  TGG T
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FIG. 1 (b)

```
  1  GTA GCT TCA TTT GTT GGC ACA GGA CTA TTC CAC CCT TAG AAT TGA CTT    48

 49  TTT GGA CAT TGA GCT AAG GTT CAA TGC ACT CGA TGG TCT TCT CAC TTC    96

 97  CGA ATA TAT AGA TCT AGC GTG TGA GGA CTA CTC ATT GGC TTG CGA TTT   144

145  ACG GTT TTT ATA TTT TTT GCC GCA CAT CAT TTT TTG GCC TGG TAT TGT   192

193  CAT CGC GGT TGA GCG GAC TCT GAA TAT AAT CCT ATT GTT TTT TAT GGA   240

241  TCT CTG GAA GCG TCT TTT TGA AGC CAA CCC AAC AAA AAT TCG AGA CAA   288

289  GAA AAT AAA AAA CGG CAC TTC ATC AGT ATC ACA AAT ACC ATC AAT TTA   336
                  A
                  ↓
337  TCA GCT CTC ATG AAT GAA ATC GAT GAG AAA AAT CAG GCC CCC GTG CAA   384
                Met Asn Glu Ile Asp Glu Lys Asn Gln Ala Pro Val Gln    13

385  CAA GAA TGC CTG AAA GAG ATG ATT CAG AAT GGG CAT GCT CGG CGT ATG   432
 14  Gln Glu Cys Leu Lys Glu Met Ile Gln Asn Gly His Ala Arg Arg Met    29

433  GGA TCT GTT GAA GAT CTG TAT GTT GCT CTC AAC AGA CAA AAC TTA TAT   480
 30  Gly Ser Val Glu Asp Leu Tyr Val Ala Leu Asn Arg Gln Asn Leu Tyr    45

481  CGA AAC TTC TGC ACA TAT GGA GAA TTG AGT GAT TAC TGT ACT AGG GAT   528
 46  Arg Asn Phe Cys Thr Tyr Gly Glu Leu Ser Asp Tyr Cys Thr Arg Asp    61

529  CAG CTC ACA TTA GCT TTG AGG GAA ATC TGC CTG AAA AAT CCA ACT CTT   576
 62  Gln Leu Thr Leu Ala Leu Arg Glu Ile Cys Leu Lys Asn Pro Thr Leu    77

577  TTA CAT ATT GTT CTA CCA ACA AGA TGG CCA AAT CAT GAA AAT TAT TAT   624
 78  Leu His Ile Val Leu Pro Thr Arg Trp Pro Asn His Glu Asn Tyr Tyr    93

625  CGC AGT TCC GAA TAC TAT TCA CGG CCA CAT CCA GTG CAT GAT TAT ATC   672
 94  Arg Ser Ser Glu Tyr Tyr Ser Arg Pro His Pro Val His Asp Tyr Ile   109

673  TCA GTA TTA CAA GAA TTG AAA CTG AGT GGT GTG GTT CTC AAT GAA CAA   720
110  Ser Val Leu Gln Glu Leu Lys Leu Ser Gly Val Val Leu Asn Glu Gln   125

721  CCT GAG TAC AGT GCA GTA ATG AAG CAA ATA TTA GAA GAA TTC AAA AAT   768
126  Pro Glu Tyr Ser Ala Val Met Lys Gln Ile Leu Glu Glu Phe Lys Asn   141

769  AGT AAG GGT TCC TAT ACT GCA AAA ATT TTT AAA CTT ACT ACC ACT TTG   816
142  Ser Lys Gly Ser Tyr Thr Ala Lys Ile Phe Lys Leu Thr Thr Thr Leu   157

817  ACT ATT CCT TAC TTT GGA CCA ACA GGA CCG AGT TGG CGG CTA ATT TGT   864
158  Thr Ile Pro Tyr Phe Gly Pro Thr Gly Pro Ser Trp Arg Leu Ile Cys   173

865  CTT CCA GAA GAG CAC ACA GAA AAG TGG AGA AAA TTT ATC TTT GTA TCT   912
174  Leu Pro Glu Glu His Thr Glu Lys Trp Arg Lys Phe Ile Phe Val Ser   189

913  AAT CAT TGC ATG TCT GAT GGT CGG TCT TCG ATC CAC TTT TTT CAT GAT   960
190  Asn His Cys Met Ser Asp Gly Arg Ser Ser Ile His Phe Phe His Asp   205

961  TTA AGA GAC GAA TTA AAT AAT ATT AAA ACT CCA CCA AAA AAA TTA GAT  1008
206  Leu Arg Asp Glu Leu Asn Asn Ile Lys Thr Pro Pro Lys Lys Leu Asp   221
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FIG. 2(a)

```
1009  TAC ATT TTC AAG TAC GAG GAG GAT TAC CAA TTA TTG AGG AAA CTT CCA   1056
 222  Tyr Ile Phe Lys Tyr Glu Glu Asp Tyr Gln Leu Leu Arg Lys Leu Pro    237

1057  GAA CCG ATC GAA AAG GTG ATA GAC TTT AGA CCA CCG TAC TTG TTT ATT   1104
 238  Glu Pro Ile Glu Lys Val Ile Asp Phe Arg Pro Pro Tyr Leu Phe Ile    253

1105  CCG AAG TCA CTT CTT TCG GGT TTC ATC TAC AAT CAT TTG AGA TTT TCT   1152
 254  Pro Lys Ser Leu Leu Ser Gly Phe Ile Tyr Asn His Leu Arg Phe Ser    269

1153  TCA AAA GGT GTC TGT ATG AGA ATG GAT GAT GTG GAA AAA ACC GAT GAT   1200
 270  Ser Lys Gly Val Cys Met Arg Met Asp Asp Val Glu Lys Thr Asp Asp    285

1201  GTT GTC ACC GAG ATC ATC AAT ATT TCA CCA ACA GAA TTT CAA GCG ATT   1248
 286  Val Val Thr Glu Ile Ile Asn Ile Ser Pro Thr Glu Phe Gln Ala Ile    301

1249  AAA GCA AAT ATT AAA TCA AAT ATC CAA GGT AAG TGT ACT ATC ACT CCG   1296
 302  Lys Ala Asn Ile Lys Ser Asn Ile Gln Gly Lys Cys Thr Ile Thr Pro    317

1297  TTT TTA CAT GTT TGT TGG TTT GTA TCT CTT CAT AAA TGG GGT AAA TTT   1344
 318  Phe Leu His Val Cys Trp Phe Val Ser Leu His Lys Trp Gly Lys Phe    333

1345  TTC AAA CCA TTG AAC TTC GAA TGG CTT ACG GAT ATT TTT ATC CCC GCA   1392
 334  Phe Lys Pro Leu Asn Phe Glu Trp Leu Thr Asp Ile Phe Ile Pro Ala    349

1393  GAT TGC CGC TCA CAA CTA CCA GAT GAT GAT GAA ATG AGA CAG ATG TAC   1440
 350  Asp Cys Arg Ser Gln Leu Pro Asp Asp Asp Glu Met Arg Gln Met Tyr    365

1441  AGA TAT GGC GCT AAC GTT GGA TTT ATT GAC TTC ACC CCC TGG ATA AGC   1488
 366  Arg Tyr Gly Ala Asn Val Gly Phe Ile Asp Phe Thr Pro Trp Ile Ser    381

1489  GAA TTT GAC ATG AAT GAT AAC AAA GAA AAT TTT TGG CCA CTT ATT GAG   1536
 382  Glu Phe Asp Met Asn Asp Asn Lys Glu Asn Phe Trp Pro Leu Ile Glu    397

1537  CAC TAC CAT GAA GTA ATT TCG GAA GCT TTA AGA AAT AAA AAG CAT CTC   1584
 398  His Tyr His Glu Val Ile Ser Glu Ala Leu Arg Asn Lys Lys His Leu    413

1585  CAT GGC TTA GGG TTC AAT ATA CAA GGC TTC GTT CAA AAA TAT GTG AAC   1632
 414  His Gly Leu Gly Phe Asn Ile Gln Gly Phe Val Gln Lys Tyr Val Asn    429

1633  ATT GAC AAG GTA ATG TGC GAT CGT GCC ATC GGG AAA AGA CGC GGA GGT   1680
 430  Ile Asp Lys Val Met Cys Asp Arg Ala Ile Gly Lys Arg Arg Gly Gly    445

1681  ACA TTG TTA AGC AAT GTA GGT CTG TTT AAT CAG TTA GAG GAG CCC GAT   1728
 446  Thr Leu Leu Ser Asn Val Gly Leu Phe Asn Gln Leu Glu Glu Pro Asp    461

1729  GCC AAA TAT TCT ATA TGC GAT TTG GCA TTT GGC CAA TTT CAA GGA TCC   1776
 462  Ala Lys Tyr Ser Ile Cys Asp Leu Ala Phe Gly Gln Phe Gln Gly Ser    477

1777  TGG CAC CAA GCA TTT TCC TTG GGT GTT TGT TCG ACT AAT GTA AAG GGG   1824
 478  Trp His Gln Ala Phe Ser Leu Gly Val Cys Ser Thr Asn Val Lys Gly    493

1825  ATG AAT ATT GTT GTT GCT TCA ACA AAG AAT GTT GTT GGT AGT CAA GAA   1872
 494  Met Asn Ile Val Val Ala Ser Thr Lys Asn Val Val Gly Ser Gln Glu    509
                                                              B
                                                              ↓
1873  TCT CTC GAA GAG CTT TGC TCC ATT TAC AAA GCT CTC CTT TTA GGC CCT   1920
 510  Ser Leu Glu Glu Leu Cys Ser Ile Tyr Lys Ala Leu Leu Leu Gly Pro    525

1921  TAG ATC TCA CAT GAT GCT TGA CTG ATA TTA TTC GAC AAT ATG ATT ATG   1968
 526  ***

1969  TCG TGT
```

FIG. 2(b)

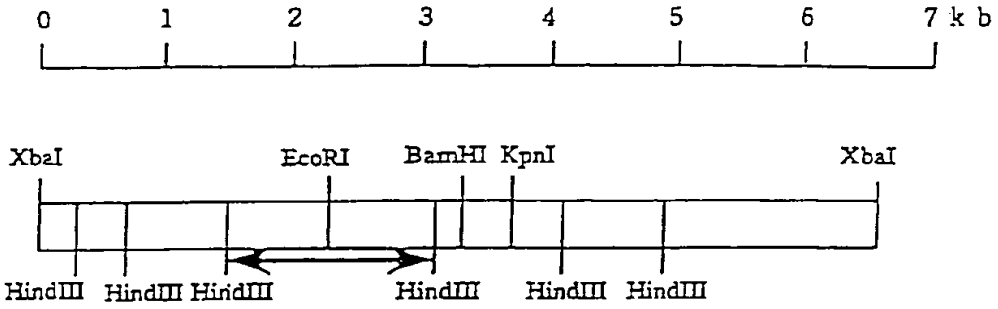

0
1
2
3
4
5
6
7 k b
XbaI
EcoRI
BamHI
KpnI
XbaI
HindIII
HindIII
HindIII
HindIII
HindIII
HindIII

FIG. 3

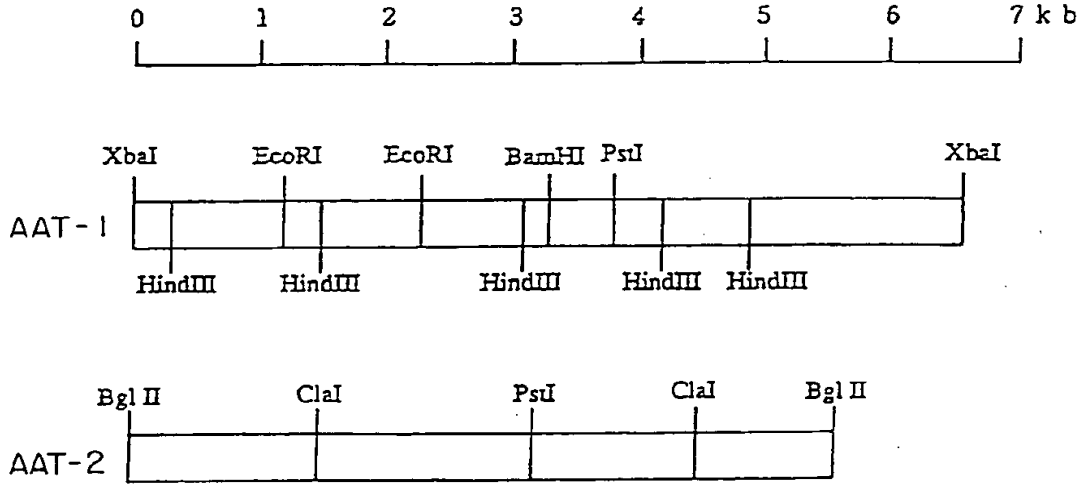

0
1
2
3
4
5
6
7 k b
XbaI
EcoRI
EcoRI
BamHI
PstI
XbaI
AAT-1
HindIII
HindIII
HindIII
HindIII
HindIII
Bgl II
ClaI
PstI
ClaI
Bgl II
AAT-2

FIG. 4

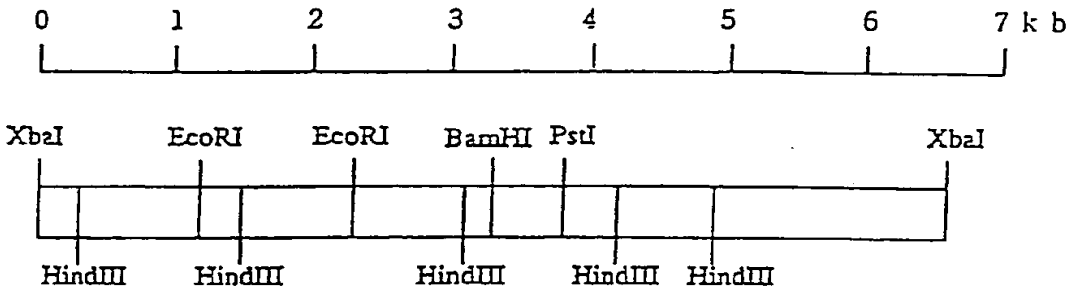
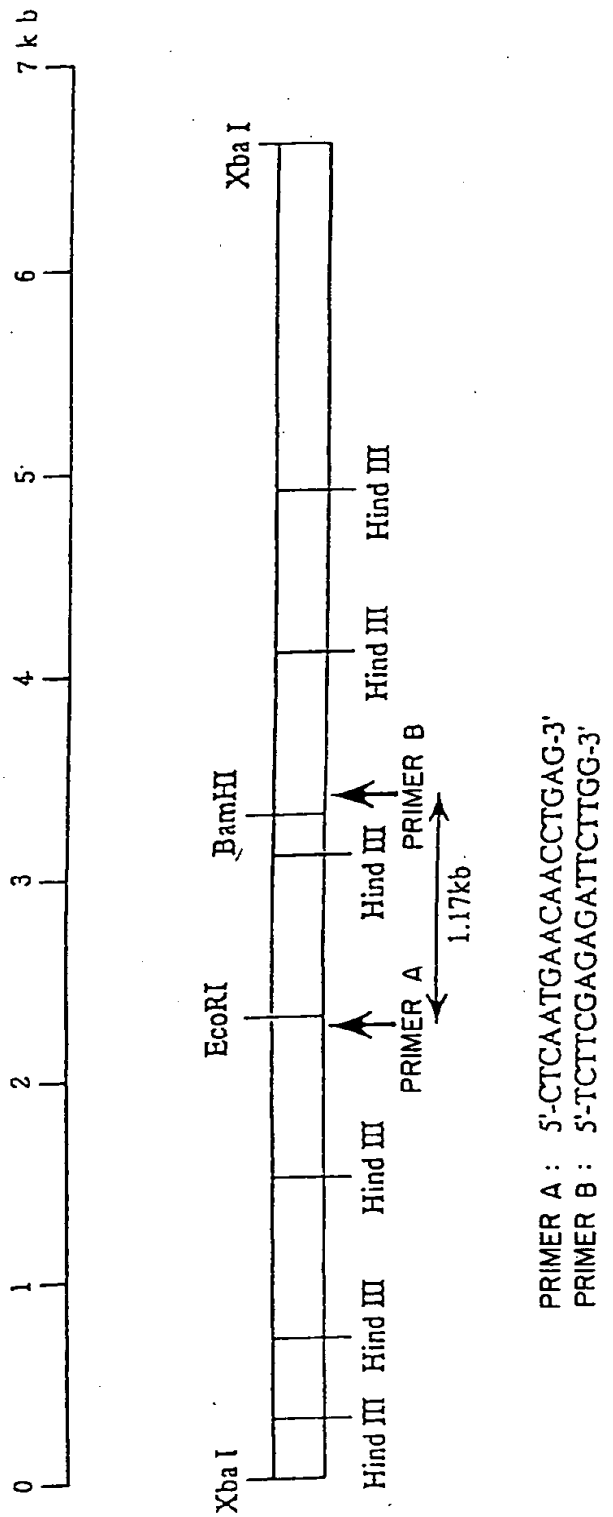

0
1
2
3
4
5
6
7 k b
XbaI
EcoRI
EcoRI
BamHI
PstI
XbaI
HindIII
HindIII
HindIII
HindIII
HindIII

FIG. 5



PRIMER A : 5'-CTCAATGAACAACCTGAG-3'
PRIMER B : 5'-TCTTCGAGAGATTCTTGG-3'

FIG. 6

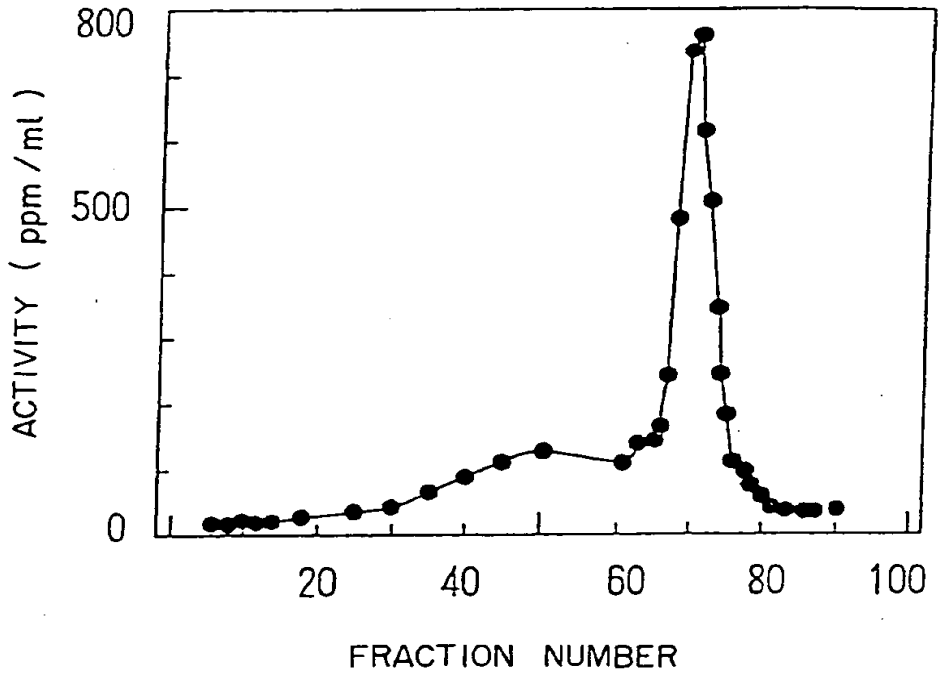


FIG. 7

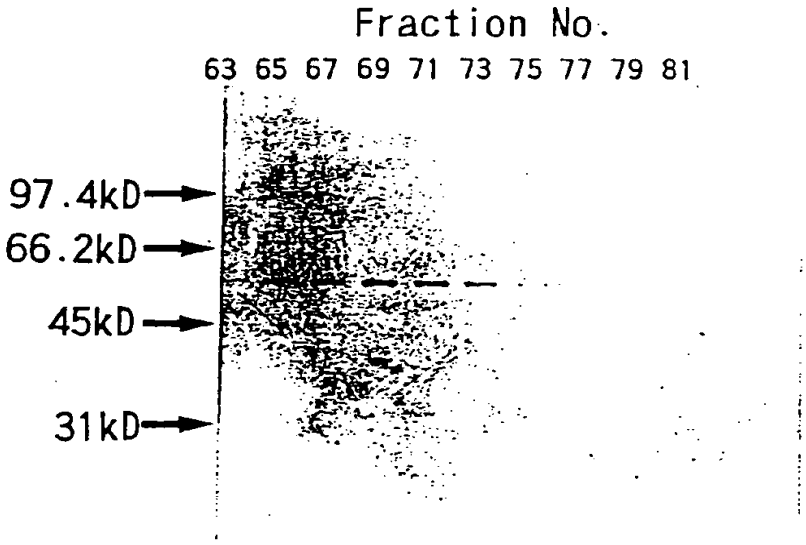


FIG. 8

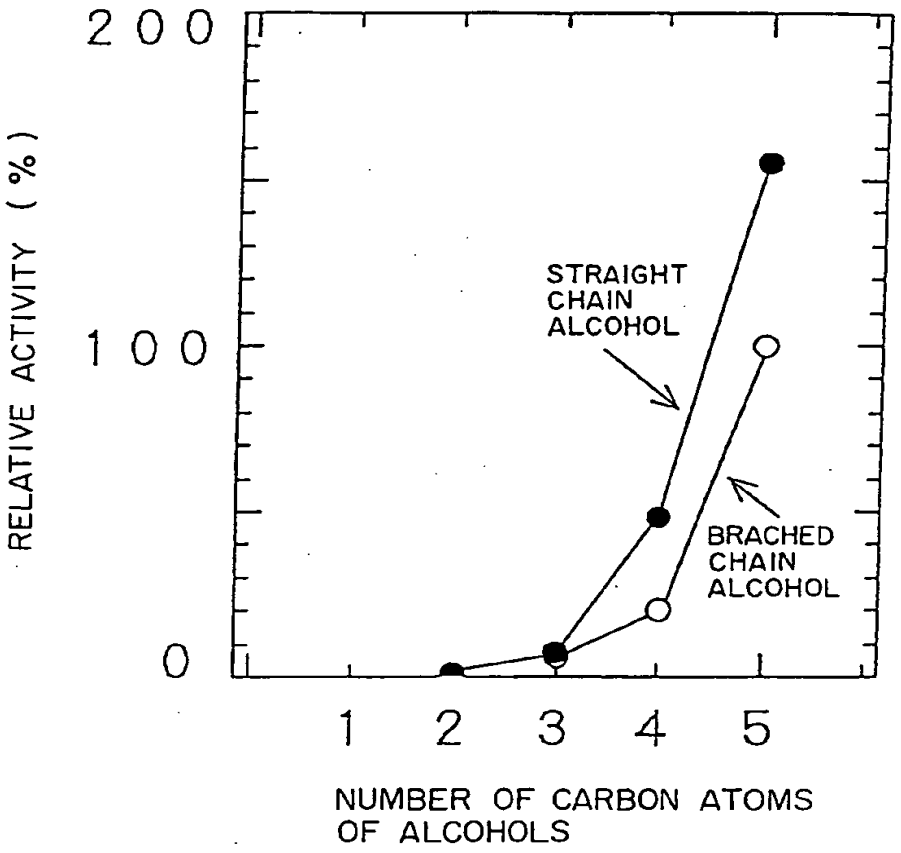

200
100
0
RELATIVE ACTIVITY (%)
STRAIGHT CHAIN ALCOHOL
BRACHED CHAIN ALCOHOL
1
2
3
4
5
NUMBER OF CARBON ATOMS OF ALCOHOLS

FIG. 9

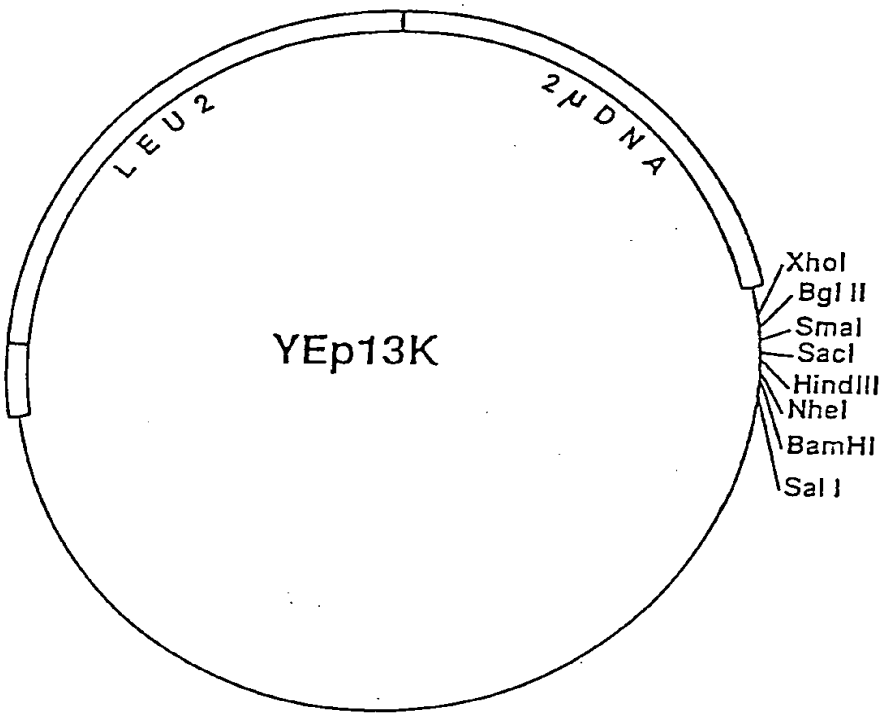

LEU2
2μ DNA
YEp13K
XhoI
BglII
SmaI
SacI
HindIII
NheI
BamHI
SalI

FIG. 10

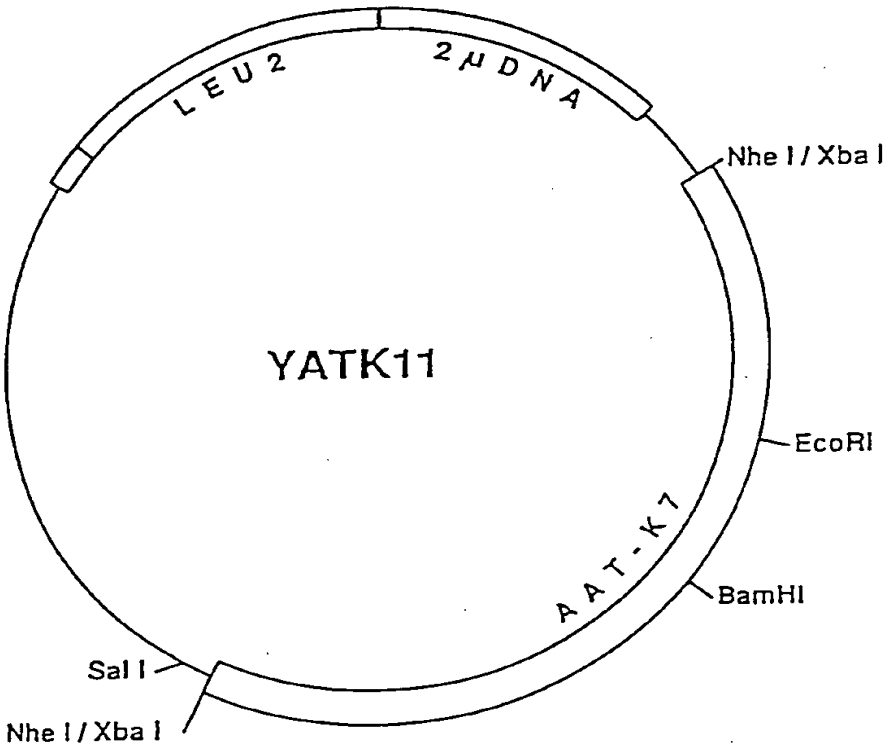

LEU2
2μDNA
Nhe I / Xba I
YATK11
EcoRI
AAT-K7
BamHI
Sal I
Nhe I / Xba I

FIG. 11

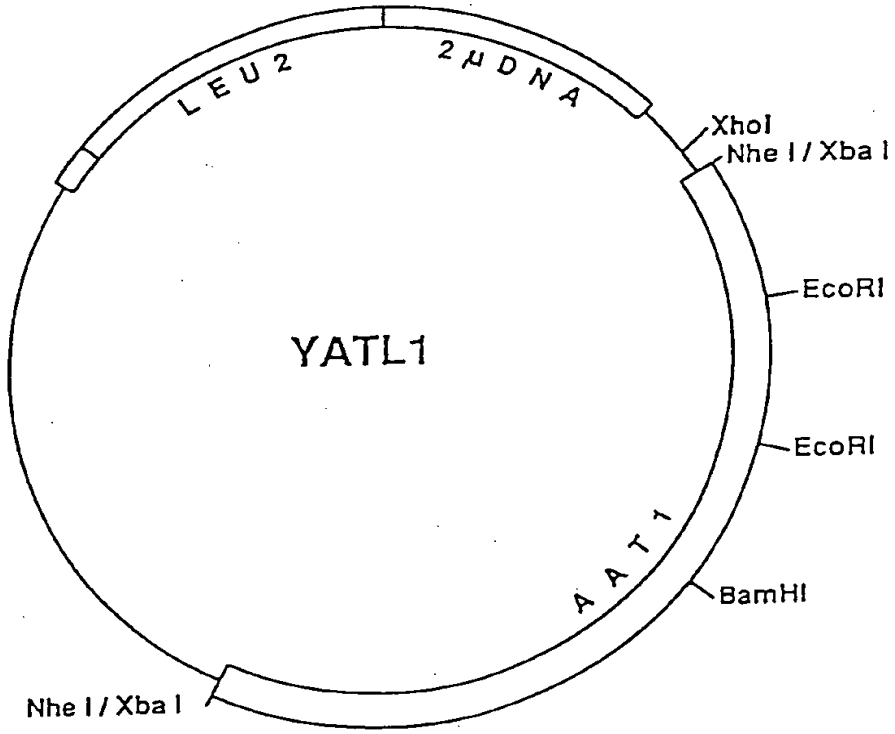

LEU2
2μDNA
XhoI
Nhe I / Xba I
EcoRI
YATL1
EcoRI
AAT1
BamHI
Nhe I / Xba I

FIG. 12

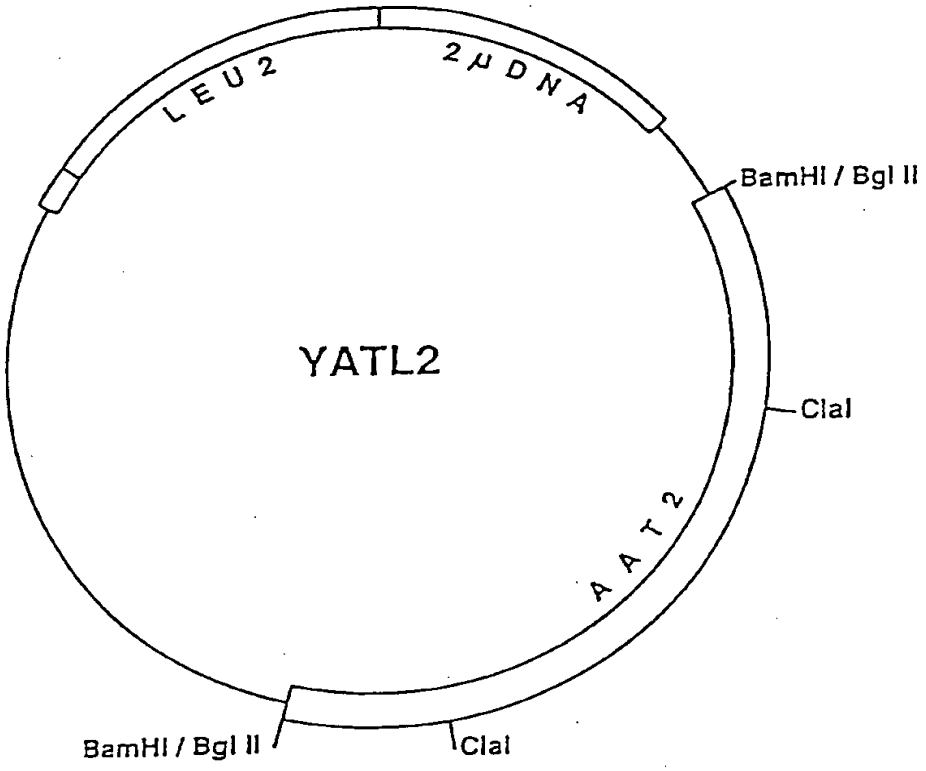


FIG. 13

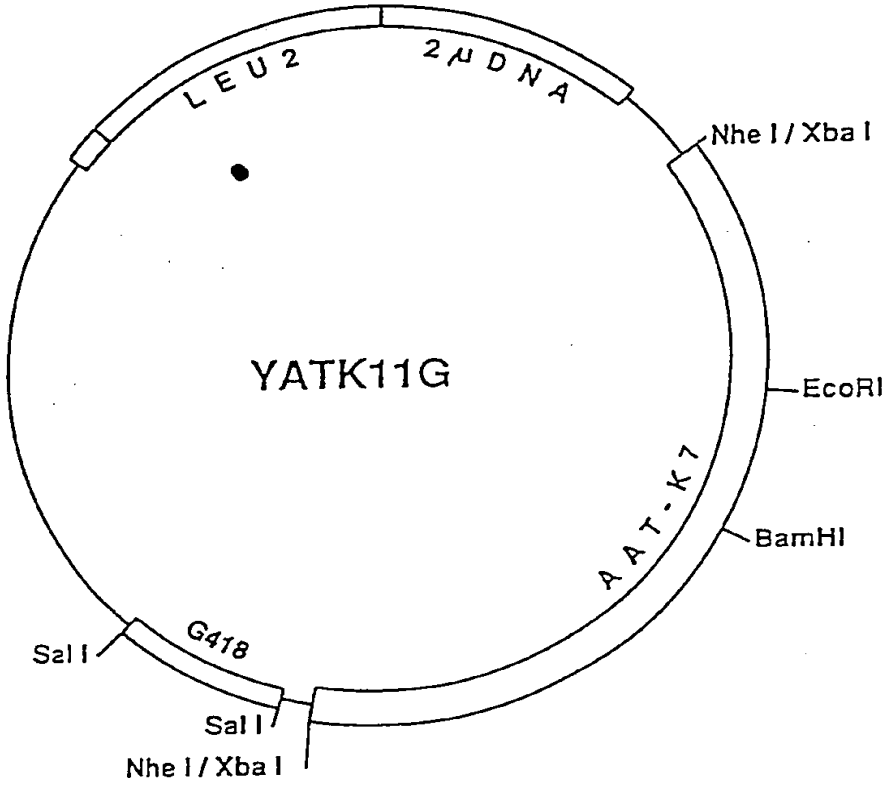

LEU2
2μ DNA
Nhe I / Xba I
YATK11G
EcoRI
AAT-K7
BamHI
G418
Sal I
Sal I
Nhe I / Xba I

FIG. 14

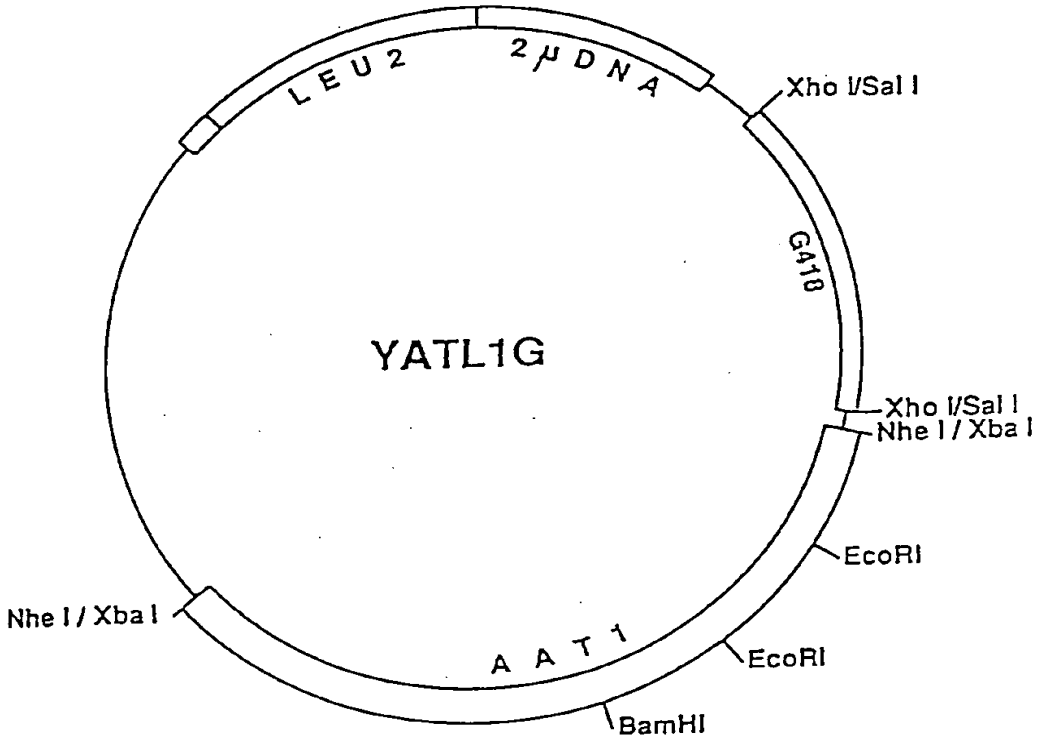

LEU2
2μDNA
Xho I/Sal I
G418
YATL1G
Xho I/Sal I
Nhe I / Xba I
EcoRI
Nhe I / Xba I
AAT1
EcoRI
BamHI

FIG. 15

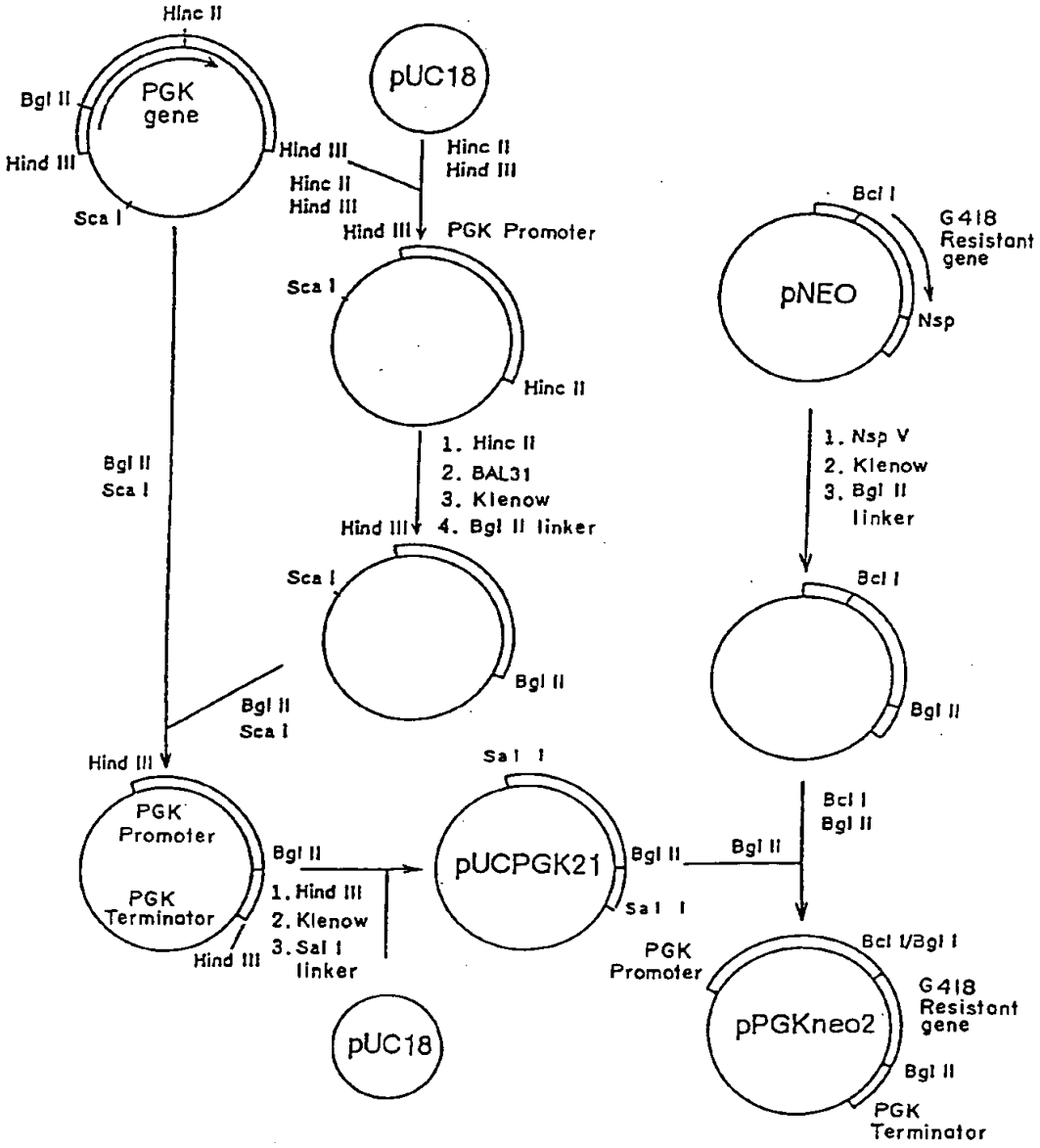

Hinc II
Bgl II
PGK gene
Hind III
Sca I
pUC18
Hinc II
Hind III
Hinc II
Hind III
Hind III
PGK Promoter
Sca I
Hinc II
1. Hinc II
2. BAL31
3. Klenow
4. Bgl II linker
Hind III
Sca I
Bgl II
Bgl II
Sca I
Hind III
PGK Promoter
PGK Terminator
Hind III
Bgl II
1. Hind III
2. Klenow
3. Sal I linker
Sal I
pUCPGK21
Bgl II
Sal I
pUC18
Bgl II
Sca I
Bcl I
G418 Resistant gene
pNEO
Nsp
1. Nsp V
2. Klenow
3. Bgl II linker
Bcl I
Bgl II
Bcl I
Bgl II
Bgl II
PGK Promoter
Bcl I/Bgl I
G418 Resistant gene
pPGKneo2
Bgl II
PGK Terminator

FIG. 16

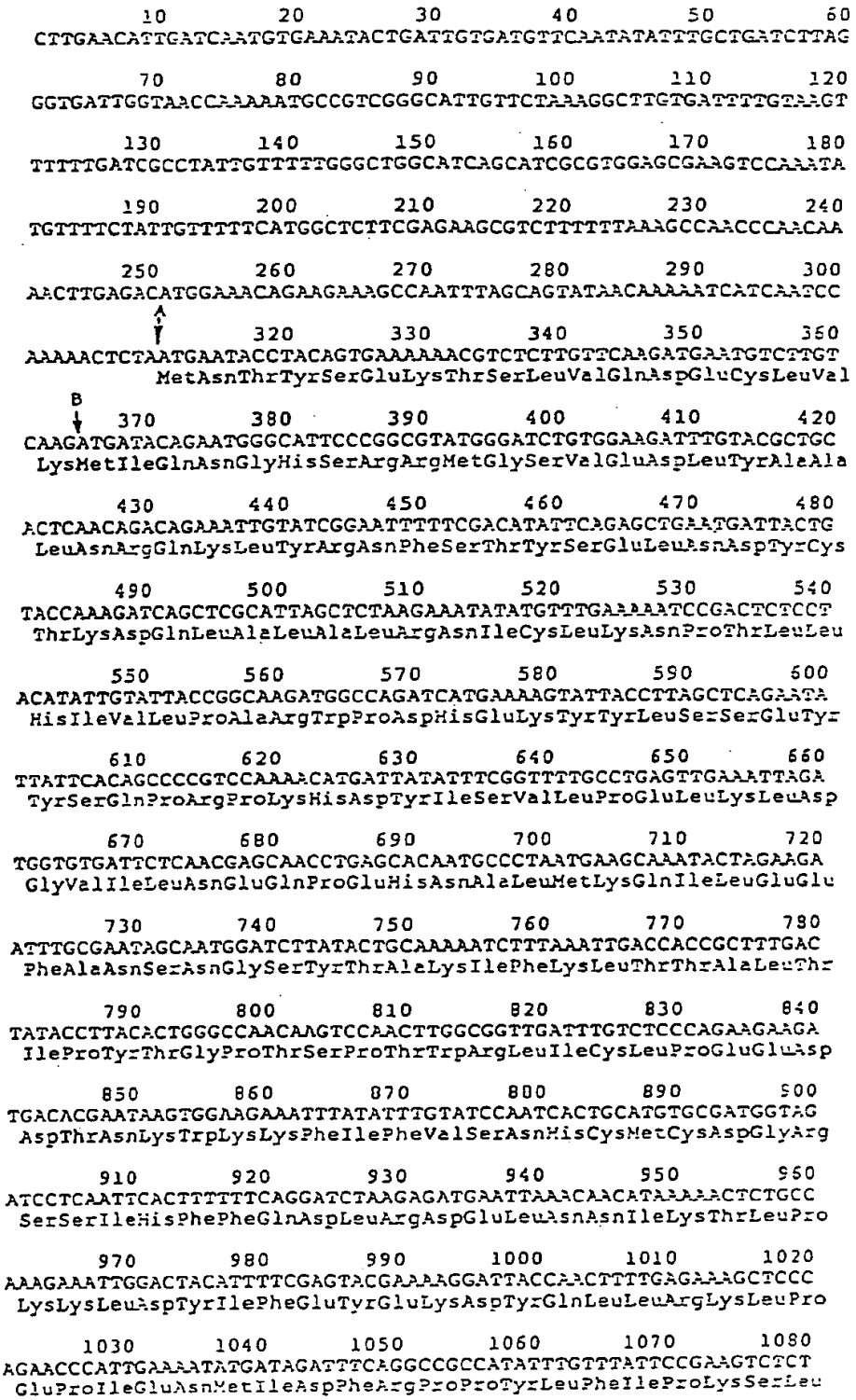

10 20 30 40 50 60
CTTGAACATTGATCAATGTGAAATACTGATTGTGATGTTCAATATATTTGCTGATCTTAG
70 80 90 100 110 120
GGTGATTGGTAACCAAAAATGCCGTCGGGCATTGTTCTAAAGGCTTGTGATTTTGTAAGT
130 140 150 160 170 180
TTTTTGATCGCCTATTGTTTTTGGGCTGGCATCAGCATCGCGTGGAGCGAAGTCCAAATA
190 200 210 220 230 240
TGTTTTCTATTGTTTTTCATGGCTCTTCGAGAAGCGTCTTTTTTAAAGCCAACCCAACAA
250 260 270 280 290 300
AACTTGAGACATGGAAACAGAAGAAAGCCAATTTAGCAGTATAACAAAAATCATCAATCC
A
320 330 340 350 360
AAAAACTCTAATGAATACCTACAGTGAAAAAACGTCTCTTGTTCAAGATGAATGTCTTGT
MetAsnThrTyrSerGluLysThrSerLeuValGlnAspGluCysLeuVal
B
370 380 390 400 410 420
CAAGATGATACAGAATGGGCATTCCCGGCGTATGGGATCTGTGGAAGATTTGTACGCTGC
LysMetIleGlnAsnGlyHisSerArgArgMetGlySerValGluAspLeuTyrAlaAla
430 440 450 460 470 480
ACTCAACAGACAGAAATTGTATCGGAATTTTTCGACATATTCAGAGCTGAATGATTACTG
LeuAsnArgGlnLysLeuTyrArgAsnPheSerThrTyrSerGluLeuAsnAspTyrCys
490 500 510 520 530 540
TACCAAAGATCAGCTCGCATTAGCTCTAAGAAATATATGTTTGAAAAATCCGACTCTCCT
ThrLysAspGlnLeuAlaLeuAlaLeuArgAsnIleCysLeuLysAsnProThrLeuLeu
550 560 570 580 590 600
ACATATTGTATTACCGGCAAGATGGCCAGATCATGAAAAGTATTACCTTAGCTCAGAATA
HisIleValLeuProAlaArgTrpProAspHisGluLysTyrTyrLeuSerSerGluTyr
610 620 630 640 650 660
TTATTCACAGCCCCGTCCAAAACATGATTATATTTCGGTTTTGCCTGAGTTGAAATTAGA
TyrSerGlnProArgProLysHisAspTyrIleSerValLeuProGluLeuLysLeuAsp
670 680 690 700 710 720
TGGTGTGATTCTCAACGAGCAACCTGAGCACAATGCCCTAATGAAGCAAATACTAGAAGA
GlyValIleLeuAsnGluGlnProGluHisAsnAlaLeuMetLysGlnIleLeuGluGlu
730 740 750 760 770 780
ATTTGCGAATAGCAATGGATCTTATACTGCAAAAATCTTTAAATTGACCACCGCTTTGAC
PheAlaAsnSerAsnGlySerTyrThrAlaLysIlePheLysLeuThrThrAlaLeuThr
790 800 810 820 830 840
TATACCTTACACTGGGCCAACAAGTCCAACTTGGCGGTTGATTTGTCTCCCAGAAGAAGA
IleProTyrThrGlyProThrSerProThrTrpArgLeuIleCysLeuProGluGluAsp
850 860 870 880 890 900
TGACACGAATAAGTGGAAGAAATTTATATTTGTATCCAATCACTGCATGTGCGATGGTAG
AspThrAsnLysTrpLysLysPheIlePheValSerAsnHisCysMetCysAspGlyArg
910 920 930 940 950 960
ATCCTCAATTCACTTTTTTCAGGATCTAAGAGATGAATTAAACAACATAAAAACTCTGCC
SerSerIleHisPhePheGlnAspLeuArgAspGluLeuAsnAsnIleLysThrLeuPro
970 980 990 1000 1010 1020
AAAGAAATTGGACTACATTTTCGAGTACGAAAAGGATTACCAACTTTTGAGAAAGCTCCC
LysLysLeuAspTyrIlePheGluTyrGluLysAspTyrGlnLeuLeuArgLysLeuPro
1030 1040 1050 1060 1070 1080
AGAACCCATTGAAAATATGATAGATTTCAGGCCGCCATATTTGTTTATTCCGAAGTCTCT
GluProIleGluAsnMetIleAspPheArgProProTyrLeuPheIleProLysSerLeu

FIG. 17(a)

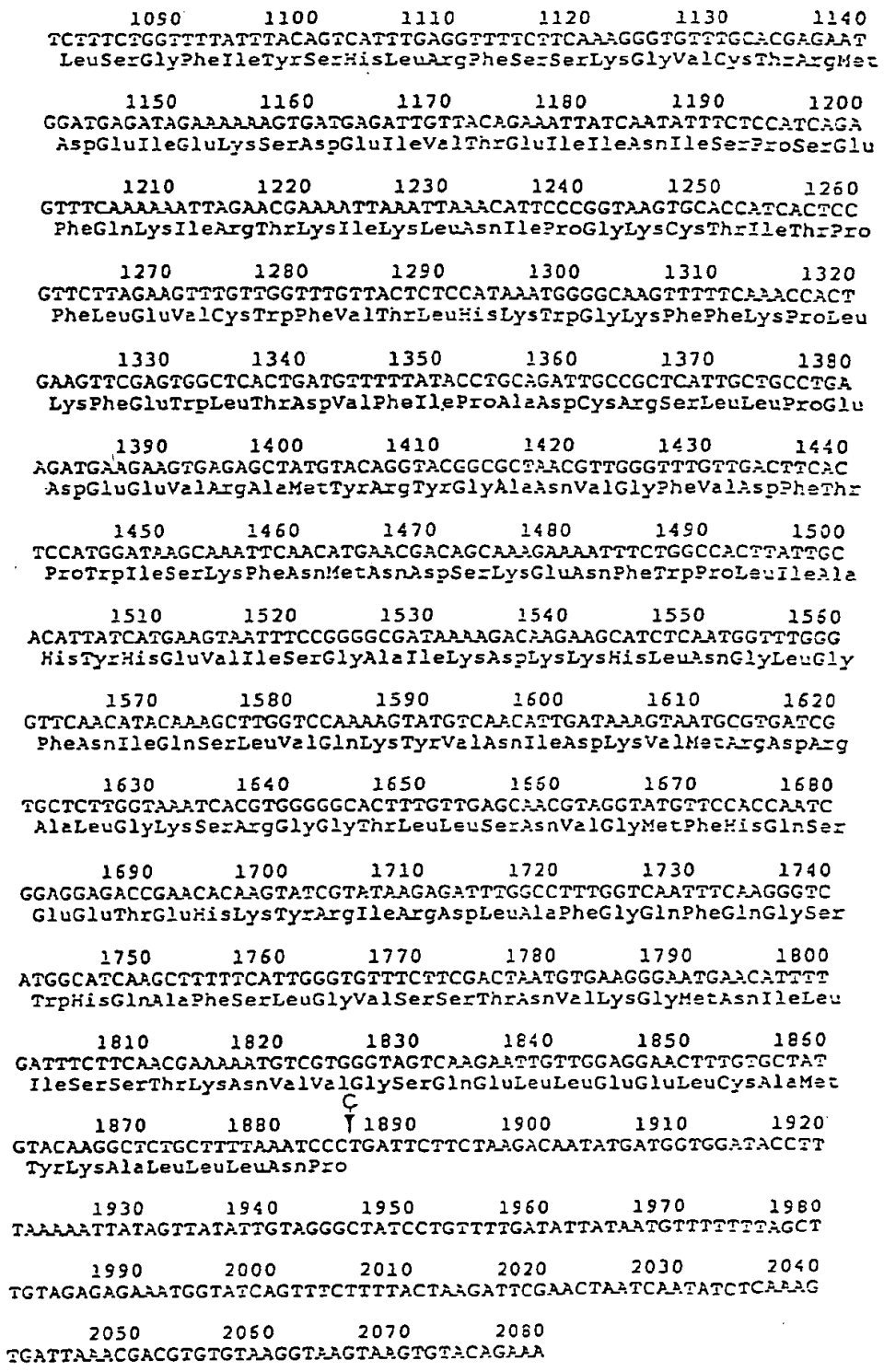

1050 1100 1110 1120 1130 1140
TCTTTCTGGTTTTATTTACAGTCATTTGAGGTTTTCTTCAAAGGGTGTTTGCACGAGAAT
LeuSerGlyPheIleTyrSerHisLeuArgPheSerSerLysGlyValCysThrArgMet
1150 1160 1170 1180 1190 1200
GGATGAGATAGAAAAAAGTGATGAGATTGTTACAGAAATTATCAATATTTCTCCATCAGA
AspGluIleGluLysSerAspGluIleValThrGluIleIleAsnIleSerProSerGlu
1210 1220 1230 1240 1250 1260
GTTTCAAAAAATTAGAACGAAAATTAAATTAAACATTCCCGGTAAGTGCACCATCACTCC
PheGlnLysIleArgThrLysIleLysLeuAsnIleProGlyLysCysThrIleThrPro
1270 1280 1290 1300 1310 1320
GTTCTTAGAAGTTTGTTGGTTTGTTACTCTCCATAAATGGGGCAAGTTTTTCAAACCACT
PheLeuGluValCysTrpPheValThrLeuHisLysTrpGlyLysPhePheLysProLeu
1330 1340 1350 1360 1370 1380
GAAGTTCGAGTGGCTCACTGATGTTTTTATACCTGCAGATTGCCGCTCATTGCTGCCTGA
LysPheGluTrpLeuThrAspValPheIleProAlaAspCysArgSerLeuLeuProGlu
1390 1400 1410 1420 1430 1440
AGATGAAGAAGTGAGAGCTATGTACAGGTACGGCGCTAACGTTGGGTTTGTTGACTTCAC
AspGluGluValArgAlaMetTyrArgTyrGlyAlaAsnValGlyPheValAspPheThr
1450 1460 1470 1480 1490 1500
TCCATGGATAAGCAAATTCAACATGAACGACAGCAAAGAAAATTTCTGGCCACTTATTGC
ProTrpIleSerLysPheAsnMetAsnAspSerLysGluAsnPheTrpProLeuIleAla
1510 1520 1530 1540 1550 1560
ACATTATCATGAAGTAATTTCCGGGGCGATAAAAGACAAGAAGCATCTCAATGGTTTGGG
HisTyrHisGluValIleSerGlyAlaIleLysAspLysLysHisLeuAsnGlyLeuGly
1570 1580 1590 1600 1610 1620
GTTCAACATACAAAGCTTGGTCCAAAAGTATGTCAACATTGATAAAGTAATGCGTGATCG
PheAsnIleGlnSerLeuValGlnLysTyrValAsnIleAspLysValMetArgAspArg
1630 1640 1650 1660 1670 1680
TGCTCTTGGTAAATCACGTGGGGGCACTTTGTTGAGCAACGTAGGTATGTTCCACCAATC
AlaLeuGlyLysSerArgGlyGlyThrLeuLeuSerAsnValGlyMetPheHisGlnSer
1690 1700 1710 1720 1730 1740
GGAGGAGACCGAACACAAGTATCGTATAAGAGATTTGGCCTTTGGTCAATTTCAAGGGTC
GluGluThrGluHisLysTyrArgIleArgAspLeuAlaPheGlyGlnPheGlnGlySer
1750 1760 1770 1780 1790 1800
ATGGCATCAAGCTTTTTCATTGGGTGTTTCTTCGACTAATGTGAAGGGAATGAACATTTT
TrpHisGlnAlaPheSerLeuGlyValSerSerThrAsnValLysGlyMetAsnIleLeu
1810 1820 1830 1840 1850 1860
GATTTCTTCAACGAAAAATGTCGTGGGTAGTCAAGAATTGTTGGAGGAACTTTGTGCTAT
IleSerSerThrLysAsnValValGlySerGlnGluLeuLeuGluGluLeuCysAlaMet
C
1870 1880 1890 1900 1910 1920
GTACAAGGCTCTGCTTTTAAATCCCTGATTCTTCTAAGACAATATGATGGTGGATACCTT
TyrLysAlaLeuLeuLeuAsnPro
1930 1940 1950 1960 1970 1980
TAAAAATTATAGTTATATTGTAGGGCTATCCTGTTTTGATATTATAATGTTTTTTTAGCT
1990 2000 2010 2020 2030 2040
TGTAGAGAGAAATGGTATCAGTTTCTTTTACTAAGATTCGAACTAATCAATATCTCAAAG
2050 2060 2070 2080
TGATTAAACGACGTGTGTAAGGTAAGTAAGTGTACAGAAA

FIG. 17(b)